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Clinical and Experimental

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VOL. 42 SEPTEMBER, 1953 No. 3

CLINICAL AND EXPERIMENTAL

THE EFFECT OF VITAMIN B_6 DEFICIENCY INDUCED BY DESOXYPYRIDOXINE IN HUMAN BEINGS

RICHARD W. VILTER, M.D., JOHN F. MUELLER, M.D., HELEN S. GLAZER, PH.D., THOMAS JARROLD, M.D., JOSEPH ABRAHAM, M.D., CARL THOMPSON, M.D., AND VIRGINIA R. HAWKINS, R.N.

CINCINNATI, OHIO

DESOXYPYRIDOXINE, a metabolic antagonist of the B_6 group of vitamins, pyridoxine, pyridoxamine, and pyridoxal, has facilitated studies on vitamin B_6 deficiency in human beings. Such a deficiency state has not been recognized under natural conditions, nor have clinical manifestations of vitamin B_6 deficiency been induced in adult subjects by dietary restrictions alone.[1] However, Snyderman, Carretero and Holt[2] have reported that infants on purified diets deficient in the vitamin B_6 group alone may develop anemia and convulsive seizures which disappear when pyridoxine is administered. Smith and Martin[3] have observed malnourished persons with cheilosis and angular stomatitis, which failed to respond to riboflavin but did respond to pyridoxine, and Spies, Bean, and Ashe[4] reported that malnourished patients with symptoms of weakness, insomnia, irritability, and difficulty in walking responded to pyridoxine after they had failed to respond to niacin, thiamin, and riboflavin. It has been presumed that vitamin B_6 is important in human nutrition, but no specific manifestations of a deficiency of this group of vitamins have been described in locales where pellagra is endemic.

Studies on microorganisms indicate that the vitamins of the B_6 group are converted to pyridoxal phosphate, a coenzyme which is important in transamination and decarboxylation reactions.[5, 6] In this role, the coenzyme facilitates the degradation of amino acids and the release and transfer of amino

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groups.[7]. The vitamin B_6 group is necessary also for normal tryptophane metabolism in laboratory animals[8] and for the conversion of this amino acid to pyridine nucleotides like cozymase.[9-13] These coenzymes are excreted as nicotinic acid metabolites such as N′-methylnicotinamide. When tryptophane metabolism is deranged because of vitamin B_6 deficiency, an abnormal intermediate metabolite, xanthurenic acid, appears in the urine,[8] and the conversion of tryptophane to the pyridine coenzymes and N′-methylnicotinamide is restricted.

There is a relationship also to fat and essential fatty acid metabolism in the rat. Fatty acids such as linoleic acid have reduced the severity and extent of pyridoxine deficiency dermatitis,[14-17] and vitamin B_6 has been shown to facilitate the utilization and interconversion of essential fatty acids.[18] The vitamin B_6 group has other important metabolic functions, one of which is a role in nucleoprotein chemistry.[19] Desoxypyridoxine phosphate, the antagonist, competes with pyridoxal phosphate, the coenzyme, and interferes with these reactions.[20]

Some of these metabolic functions have been investigated in human beings under various physiologic and pathologic conditions. Biochemical tests have uncovered some evidence for human vitamin B_6 insufficiency. Spies, Ladisch, and Bean[21] noted that chronically malnourished persons excreted in the urine only a small percentage of administered pyridoxine as compared to adequately nourished persons. Greenberg, Bohr, McGrath, and Rinehart[22] have reported that subjects maintained on a diet deficient in the vitamin B_6 complex excreted large amounts of xanthurenic acid in the urine after a test dose of tryptophane. A similar abnormality in the metabolism of tryptophane has been reported by Wachstein and Gudaitis[23] in women in the second and third trimesters of pregnancy and by Sprince, Lowy, Folsome and Behrman[24] in pre-eclamptic and eclamptic women. In each instance the administration of pyridoxine overcame the abnormality, and xanthurenic acid excretion was strikingly reduced. Studies on alanine and urea nitrogen metabolism in pregnant women support the hypothesis that either a dietary or a conditioned deficiency of the vitamin B_6 group occurs in this physiologic state and may be accentuated during pernicious vomiting of pregnancy.[25] These deviations from the normal are rectified by pyridoxine, probably because deamination and transamination reactions are improved by the vitamin.

In 1950, Mueller and Vilter[26] reported the clinical manifestations of vitamin B_6 deficiency observed in eight human subjects treated with desoxypyridoxine and a diet poor in the vitamins of the B complex. Seborrheic dermatitis, glossitis, cheilosis, and lymphopenia were described. Later Glazer, Mueller, Thompson, Hawkins, and Vilter[27] reported that xanthurenic acid appeared in the urine of these subjects after a test dose of *d* or *dl* tryptophane, but that N′-methylnicotinamide excretion after tryptophane was unaffected.[28] Since these reports, fifty patients have been studied, including the eight reported in the first paper. Numerous clinical and biochemical tests have been performed to assess metabolic functions during the deficiency state. These studies which are embodied in the following report indicate that vitamin B_6 deficiency in

human beings mimics the clinical manifestations of all vitamin B complex deficiency states and that the vitamin B_6 group is involved in the metabolism of tryptophane, other amino acids, the pyridine nucleotides, and possibly the essential fatty acids.

MATERIAL AND METHODS

Thirty-eight white and twelve Negro patients with a wide variety of chronic diseases listed in Table I agreed to take desoxypyridoxine. The antimetabolite was administered for three to fifty-two weeks. The doses ranged from 50 to 500 mg. daily. The oral route was used in two-thirds of the patients after it was shown that this method of administration was as effective as the parenteral route. Thirty were given vitamin B complex poor diets;* twenty were on regular hospital diets. Thirty-eight were men and twelve were women. The ages of the patients ranged from 21 to 87 years. Thirty-one were over the age of 50 years.

Each patient was examined thoroughly for signs of nutritional deficiency disease before the experimental program began. All patients were in the hospital and were observed daily by a member of the resident staff and twice weekly or oftener by a member of the research team. During experiments that involved production of acute deficiency disease, the patients were observed daily or oftener by a member of the team after symptoms or signs appeared. The experiment with chronic deficiency states did not require more frequent observation than the twice weekly routine. The following tests were performed on groups of these patients before the experimental period, during the induction of the deficiency state, during the period of symptomatic deficiency disease, and after one of the group of B_6 vitamins had relieved the clinical manifestations.

Erythrocyte counts, hemoglobin levels, white blood cell counts, differential white blood cell counts, reticulocyte counts, and platelet counts were done on 18 patients by standard techniques and with equipment certified by the U. S. Bureau of Standards. Bone marrow was aspirated from the sternum or iliac crests in five patients, and coverslip films were stained with Wright-Giemsa technique.

TABLE I. PRINCIPAL DIAGNOSES IN THE 50 PATIENTS WHO RECEIVED DESOXYPYRIDOXINE

NO. OF PATIENTS	DIAGNOSIS
1	Healed miliary tuberculosis
6	Cerebrovascular accident
1	Bronchogenic carcinoma
4	Multiple sclerosis
6	Rheumatoid arthritis
2	Paraplegia or quadriplegia
1	Amyotrophic lateral sclerosis
3	Tabes dorsalis
1	Fractured leg
1	Osteoarthritis
8	Paralysis agitans
1	Myeloid leukemia (subacute)
4	Lymphatic leukemia (chronic)
1	Bronchial asthma
1	Osteoporosis
1	Syphilitic encephalomalacia
2	Paresis
1	Cerebral arteriosclerosis
1	Friedreich's ataxia
1	Hysteria
2	Controlled hyperthyroidism
1	Gout
50	

*The deficient diet had the following composition: calories 3,100, protein 31 Gm., fat 27 Gm., thiamin 0.42 mg., niacin 4.2 mg., riboflavin 0.60 mg., (calculated from Bowes and Church, 1946) and pyridoxine 0.5 mg. (estimated).

Eosinophils, total white blood cells, lymphocytes, and neutrophils were determined in six patients before and at two- and four-hour intervals after the intramuscular injection of ACTH. The subjects were kept in bed and did not eat during these tests.

Xanthurenic acid was measured[27] in twenty-four hour urine collections from thirty-three subjects before and after a test dose of 5 Gm. of *l* tryptophane or 10 Gm. of *dl* tryptophane.* N′-methylnicotinamide was measured[29] in the urine specimens from seven of these patients and sometimes for an additional twenty-four hour period. Urine urea nitrogen[30] and uric acid[31] were measured in one group of three subjects.

The following chemical tests were performed on the blood of five subjects: fasting sugar levels,[32] intravenous and oral glucose tolerance tests, serum protein, albumin-globulin ratios,[33] urea nitrogen,[30] uric acid,[34] blood lipids,[35] and cholesterol, free and esterified[36] before and after a high fat, high cholesterol meal. A patient on the diet low in the vitamins of the B complex served as a control for each of the tests listed above.

In addition blood urea nitrogen levels were determined in three patients before and twelve hours after a test dose of thirty grams of alanine given orally.[25] The alanine was administered in fruit juice at 8 P.M., and the patient received no food until a blood specimen was collected at 8 A.M. the next morning. In another group of four patients tests were performed on the effect of the B complex poor diet supplemented with 100 Gm. of vitamin poor casein on symptoms, signs, xanthurenic acid, and N′-methylnicotinamide excretion.

The effect of 25 mg. thiamin, 100 mg. niacin, and 10 mg. riboflavin were noted on each type of lesion observed. In some patients these vitamins were given before the lesions appeared; in others, after the lesions had become florid. Two patients with glossitis and stomatitis received 25 to 50 mg. of cozymase by slow intravenous drip in 500 ml. of 5 per cent glucose daily for four to five days after 300 mg. of niacin amide had not altered the lesions.

Four patients with dermatitis were treated with 4 to 12 Gm. of Linobione† (linoleic acid) by the oral route daily for three weeks to three months. Five patients with dermatitis were treated with 1 per cent pyridoxine ointment applied to a localized skin lesion. A similar lesion, symmetrically placed, was treated with the ointment base (neobase or vanibase).

Finally, five patients have been maintained in a state of chronic vitamin B_6 deficiency for twelve months. The dose of desoxypyridoxine has ranged from 75 to 300 mg. daily and has been reduced or increased within these limits in order to maintain a low-grade deficiency state without severe lesions. From time to time, the tests mentioned above have been repeated.

RESULTS

Clinical Observations.—Of the fifty patients who received desoxypyridoxine, thirty-four developed symptoms and signs of vitamin B_6 deficiency. It is impossible to state with accuracy the dose of desoxypyridoxine which is required to induce signs of this deficiency in any one patient. Apparently each batch of desoxypyridoxine differs in the amount of pyridoxine which contaminates it, so each batch differs in potency. We have had several batches which failed to induce any sign of vitamin B_6 lack after months of trial, and other lots which were extremely potent. In general, patients on a diet deficient in vitamins of the B complex develop lesions more frequently and in a shorter period of time than patients on a regular hospital diet. For instance, with one potent lot of desoxypyridoxine given in doses of fifty to 199 mg. daily only 33 per cent of patients on a regular diet developed any lesions after a period

*Supplied by Dr. Robert Peterman of Merck and Company, Inc., Rahway, N. J.
†Linobione was supplied by Gelatin Products Division, R. P. Scherer Corp., Detroit, Mich.

of sixty days, while 86 per cent of patients on a diet poor in B complex vitamins developed lesions in the same length of time. With a less potent lot of desoxypyridoxine given in doses of 200 to 299 mg. daily, 50 per cent of patients on a regular diet and 75 per cent on a B complex poor diet developed symptoms and signs after ninety days. Lesions have appeared in as little time as five days after desoxypyridoxine administration is begun, and occasionally will not develop even after a year of such treatment. The average length of time required is twenty-two days.

Seborrheic dermatitis, indistinguishable from seborrhea sicca, is the most common lesion. It begins as an area of erythema usually in the nasolabial folds, but sometimes around the eyes, in the eyebrows, and at the angles of the mouth. Itching and burning sensations are associated with the erythema,

Fig. 1.—Dermatitis of the seborrhea sicca type due to pyridoxine deficiency induced by desoxypyridoxine. The skin is red and rough in the nasolabial folds and on the chin and the greasy scales are clearly visible.

and within twenty-four hours the involved areas are covered with yellowish oily scales. Frequently the lesion spreads from the area involved initially and has been observed over the entire face, behind the ears, in the scalp, down the neck, over the shoulders, in the perineal region, and over the buttocks. This lesion was the first sign of pyridoxine deficiency in 24 patients, and eventually appeared in 29. (See Figs. 1 and 2.)

Glossitis indistinguishable from that of acute niacin deficiency occurred in five patients as the initial manifestation of pyridoxine deficiency, and ultimately appeared in fourteen before the experimental period was terminated by the administration of pyridoxine. At first the tongue burns as if scalded by hot coffee. Usually within the next twenty-four hours the papillae at the

tip of the tongue are red, or areas of red hypertrophied filiform papillae may be found on the sides or dorsal surface of the tongue. Later the tongue swells sufficiently to blot out the structure of the filiform papillae, which remain as red dots. The fungiform papillae stand out as hypertrophied red knobs. (See Fig. 3.)

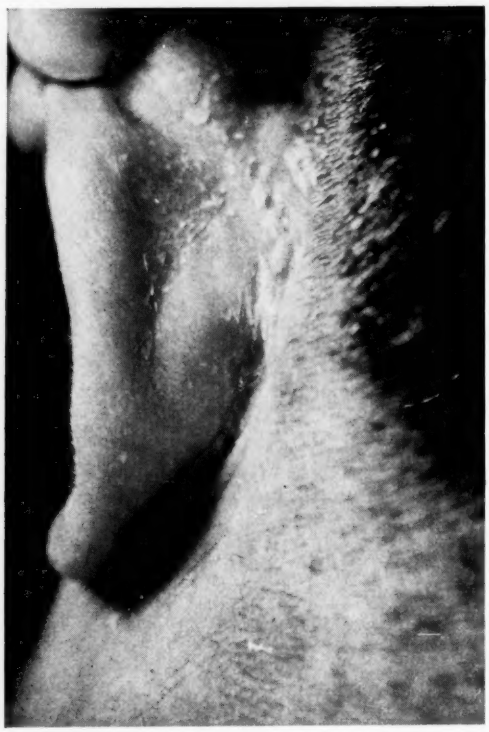

Fig. 2.—Seborrhea sicca behind the ear of a patient with pyridoxine deficiency induced by desoxypyridoxine.

The oral mucous membrane becomes reddened diffusely or focally. Small ulcerations may develop on this reddened background. This lesion is similar to niacin deficiency stomatitis and often resembles the lesions of mild aminopterin toxicity. (See Fig. 4.)

Cheilosis, angular stomatitis, and conjunctivitis similar to the lesions of riboflavin deficiency occurred as the initial clinical manifestations in three patients. (See Fig. 5.) Before the test periods were terminated, six patients had conjunctivitis. Keratitis was not observed.

Severe sensory neuritis was the initial manifestation of vitamin B_6 lack in one patient. Two others developed this lesion before the termination of the experiment. It began as a sensation of tingling and numbness in the hands and feet. In one patient no further progression was noted, but in two the lesion progressed rapidly in ascending fashion. Within four to five days the hands, arms, legs, and feet were hyperesthetic to pinprick. Afterglow was a

prominent sign. Perception of vibration and the position of the toes and fingers was greatly impaired, and the deep tendon reflexes became hypoactive. Plantar reversal signs were not observed. After treatment with pyridoxine had eliminated all physical signs of neuritis, these patients experienced the "hot foot" or "electric foot" syndrome described so frequently by prisoners of war in the Pacific area.[37, 38] Nighttime was the most troublesome period. Subjective sensations of burning and electric shocks, particularly in the feet, forced these patients to kick the bed covers off. Gradually over a period of several months, these symptoms subsided.

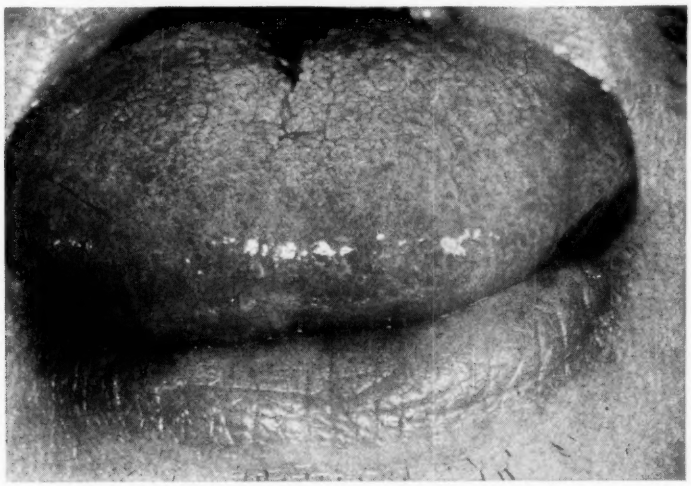

Fig. 3.—Acute glossitis in a patient with pyridoxine deficiency induced by desoxypyridoxine. The tongue is very red and smooth, especially along the edges. The filiform papillae are flattened and red; the fungiform papillae are hypertrophied.

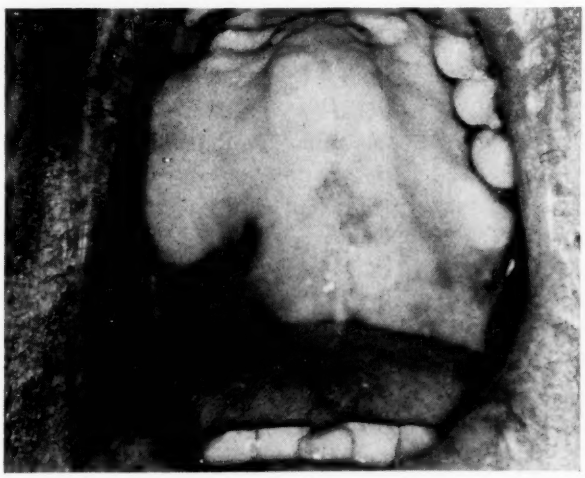

Fig. 4.—Stomatitis in a patient with pyridoxine deficiency induced by desoxypyridoxine. The dark areas on the palate are spots of acute erythema. The light background is the normal mucosa.

Two patients developed erythema of the dorsal surfaces of the arms and legs which spread on to the ventral surfaces. In one of these patients the erythema involved the neck in collar distribution and the perineal area. Pigmentation and scaling followed the erythema, and the lesion assumed the morphologic characteristics of chronic pellagrous dermatitis. (See Fig. 6.) One of these patients had two separate trials on desoxypyridoxine. On both occasions this same type of dermatitis appeared.

Two patients developed intertrigo in the perineal and inguinal regions, under the breasts, and in the axillae. These lesions were resistant to the usual types of local therapy until pyridoxine was administered. Then they healed rapidly.

Fig. 5.—Cheilosis, a reddened, sore fissured lip, in a patient with pyridoxine deficiency due to desoxypyridoxine.

Anorexia and drowsiness were the most common subjective manifestations of the deficiency state. Thirteen patients developed moderate degrees of nausea and vomiting. Eight became lethargic, somnolent, and confused. Six patients who were prone to infections because of pre-existing medical disease developed pneumonia, pyelonephritis, or superficial skin infections. In one of these, a patient with paraplegia who developed pyelonephritis, the therapeutic response was poor with antibiotics active against the offending organism in vitro. These same antibiotics were effective after the deficiency state was relieved by pyridoxine. These observations are reminiscent of similar ones by Stoerk in pyridoxine deficient rats.[39, 40] Weight loss was common after deficiency signs appeared. Some patients lost as much as 15 pounds.

Five patients who have received 75 to 300 mg. of desoxypyridoxine for fifty-two weeks developed the same symptoms and signs as those described above. The lesions were mild and qualitatively the same. No evidence was found to indicate that chronic vitamin B_6 deficiency differed from the acute state.

Observations were made (also) of the effect of the deficiency state on certain chronic diseases which the subjects had. No changes were observed in chronic myeloid leukemia or paralysis agitans, diseases which pyridoxine reportedly has benefited.[41, 42] Lymph nodes and spleen decreased in size in patients with chronic lymphatic leukemia, but no effect was observed on the circulating lymphocytes. These results are much less striking than those obtained by Weir.[43] Patients with acute childhood leukemia, lymphocytic lymphoma, and multiple myeloma were not benefited. One patient with rheumatoid arthritis had psoriasis. The psoriatic lesions spread dramatically during the deficiency state, and then regressed after treatment with pyridoxine was instituted.

Fig. 6.—Scaling, hyperpigmented skin lesions on the arms around the elbows in a patient with pyridoxine deficiency due to desoxypyridoxine.

Therapeutic Tests.—In all instances when treatment with any specific nutrient was begun, the regime which had induced the lesions was continued without any other change. Seven patients received the vitamin mixture containing thiamin 25 mg., niacin 100 mg., and riboflavin 10 mg., intramuscularly for five days after lesions had appeared. No improvement occurred in the dermatitis, glossitis, or cheilosis. Three patients received these vitamins intramuscularly before the appearance of lesions. No interference was noted with the development of these lesions. The two patients who developed neuritis were treated with thiamin 50 mg. twice daily orally for four to five days before any additional therapy. The neuritis progressed during this period.

One patient with glossitis received 300 mg. of nicotinamide daily for five days without improvement in his glossitis. Thereafter he was given 50 mg. cozymase (Nutritional Biochemicals) in 500 ml. of 5 per cent glucose by slow intravenous drip daily for four days. The burning sensations in his tongue and oral mucous membranes had disappeared in twenty-four hours, and in forty-eight hours tongue and mouth were normal. Another patient with a

similar lesion was treated with cozymase, 25 mg. daily by intravenous drip, without prior nicotinamide therapy. The glossitis and stomatitis disappeared in the same length of time.

Two patients with seborrheic dermatitis and intertrigo, and two with seborrheic dermatitis have been treated with capsules of linoleic acid (Linobione) in doses of 4, 8, or 12 Gm. daily for three weeks to three months. Improvement was noted in two patients. A man, who received 8 Gm. daily, had extensive dermatitis on face and perineum. After a period of four weeks the skin lesions had disappeared, but glossitis and neuritis were not affected. The second patient, a woman with severe intertrigo and mild facial dermatitis improved on 12 Gm. daily. The patients on the low doses showed no improvement.

After these clinical trials and other special tests had been performed, each patient received pyridoxine, pyridoxamine, or pyridoxal in doses of 5 to 200 mg. daily either orally or parenterally. Each member of the vitamin B_6 complex was equally effective, and there was no difference between oral or parenteral administration. The low doses appeared to be as effective as the higher ones.

Within twelve hours the patients felt better, nausea disappeared, and appetite improved. In twenty-four hours, burning sensations of the tongue and oral mucous membranes had disappeared and erythema had begun to fade. In forty-eight hours the tongue and mouth were normal, and those who had neuritis noted subjective improvement. All skin lesions had cleared between the third and eighth days. Cheilosis, angular stomatitis, and conjunctivitis had disappeared by the fourth or fifth days. Objective evidence of neuritis gradually receded after the fourth day, and had disappeared by the twenty-first day. The "hot foot" syndrome continued for several months.

A 1 per cent pyridoxine ointment in vanibase was applied three times daily to a facial lesion in five of these patients. In each patient the ointment base was applied to a symmetrically placed lesion on the opposite side of the face. In three of these patients the lesion treated was in the nasolabial fold and upper lip; in one, in the eyebrow. Such small lesions treated with pyridoxine ointment cleared in forty-eight to seventy-two hours, though the symmetrical control area was unaffected. One patient, half of whose face was treated with pyridoxine ointment, recovered rapidly from all manifestation of pyridoxine deficiency.

Mild lesions in three patients disappeared spontaneously, even though there was no change in diet or desoxypyridoxine. In one patient skin erythema disappeared, though glossitis progressed until pyridoxine was administered. Apparently spontaneous partial remissions and exacerbations were noted in patients with glossal and dermal lesions who were kept on the regime for many months. These swings in severity occurred over several weeks. In this respect also the induced deficiency state mimicked naturally occurring deficiency diseases.

Hematologic Observations.—Eighteen patients who developed lesions of vitamin B_6 deficiency were studied hematologically for three weeks to twelve months. Mild normocytic normochromic anemia developed in seven patients. The maximum fall in erythrocytes was from 4.5 million to 3.4 million cells in four weeks. Hemoglobin diminished proportionately. The average decrease in erythrocytes and hemoglobin in these seven patients was 400,000 cells and 1½ Gm., respectively. No diagnostic abnormalities were found in the bone marrow of the five patients from this group on whom the examination was

EFFECT OF PYRIDOXINE DEFICIENCY INDUCED BY DESOXYPYRIDOXINE ON THE TOTAL LYMPHOCYTE COUNTS IN 18 PATIENTS

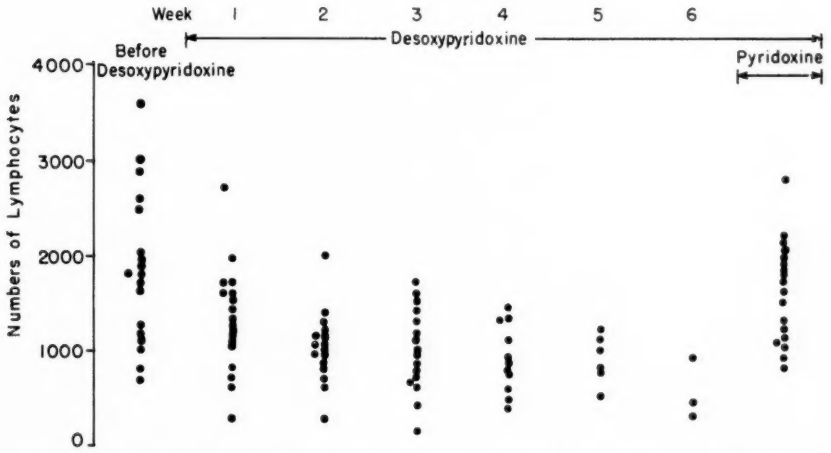

Fig. 7.—This chart demonstrates the absolute lymphopenia which develops in patients with pyridoxine deficiency induced by desoxypyridoxine. Each dot represents the absolute lymphocyte count of one patient.

performed. Reticulocytosis did not occur after pyridoxine was administered, and erythrocytes and hemoglobin rose slowly over a period of two months. No definite relationship of the anemia to vitamin B_6 deficiency could be established.

Total white blood cell counts did not change significantly. Seventeen of the eighteen patients developed absolute lymphopenia, usually at the time that deficiency signs appeared. (Fig. 7.) Clear-cut polymorphonuclear leukocytosis did not develop, though in ten patients the neutrophil counts tended to be higher during the deficiency state than before or afterward. (Fig. 8.) In those patients who had widespread skin lesions, there was eosinophilia. The number of monocytes varied so greatly that no clear picture could be established.

Within a week after the administration of pyridoxine the lymphocytes and eosinophils returned to pretreatment numbers. No change in any of the peripheral blood elements were found in six patients who received desoxypyridoxine, but developed neither symptoms nor signs of vitamin B_6 lack.

Biochemical Observations.—The metabolism of tryptophane: Of the thirty-four patients who received test doses of tryptophane before, during, and after the period of desoxypyridoxine administration, thirty excreted large amounts of xanthurenic acid in the urine during the deficiency period (range 56 to 500 mg. per twenty-four hours). Normal values (under 30 mg. per twenty-four hours) were usually found before desoxypyridoxine was given and within five

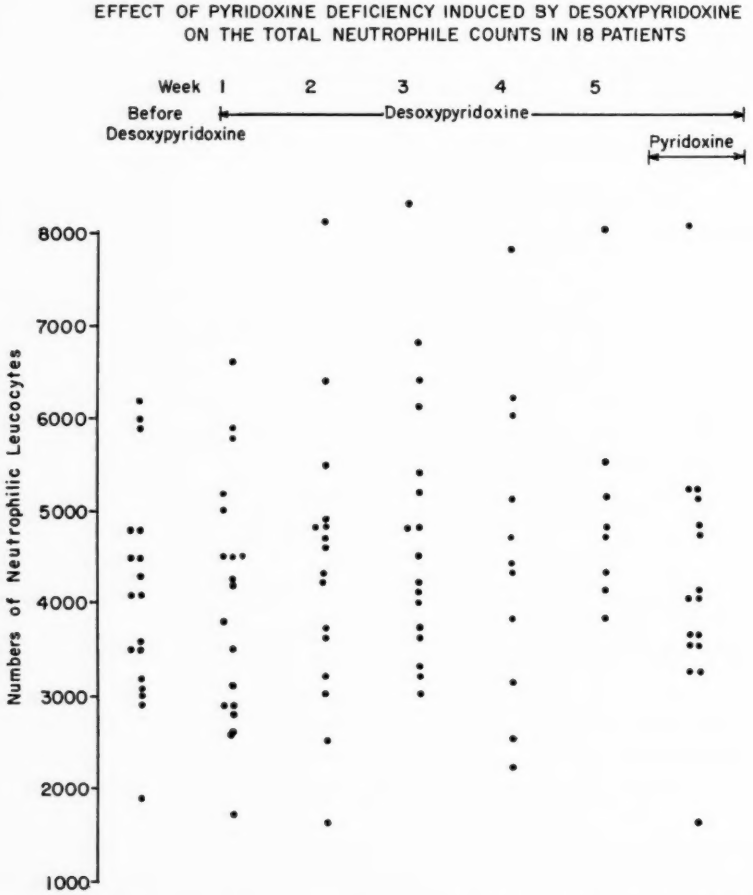

Fig. 8.—This scatter graph demonstrates little effect of pyridoxine deficiency induced by desoxypyridoxine upon the total number of neutrophiles. Each dot represents the total neutrophile count in one patient.

days after the inclusion of pyridoxine in the regime whether given orally, parenterally, or applied to the skin as a 1 per cent ointment. Usually xanthurenic acid increased in the urine long before symptoms or signs were manifest. In thirteen patients the xanthurenic acid increased five days to eight months before any lesion. In very acute experiments on three patients the increase in xanthurenic acid and the clinical manifestation occurred simultaneously. In four patients xanthurenic acid increased after the dermatitis

was well established. In eleven subjects who excreted abnormally large amounts of xanthurenic acid, clinical deficiency signs or symptoms never appeared, probably because the experiments were not continued for a sufficient length of time. Three subjects did not excrete more than a normal amount of xanthurenic acid nor did they develop symptoms or signs of vitamin B_6 lack. There was no correlation between the amount of xanthurenic acid that was found and the severity or type of symptom or sign. (See Table II.)

The amount of N′-methylnicotinamide in the urine was determined in seven of the patients who excreted abnormal amounts of xanthurenic acid. These tests were performed twenty-four hours before, and twenty-four and forty-eight hours after the administration of tryptophane. No difference in

TABLE II. THE OCCURRENCE OF XANTHURENIC ACID IN THE URINE AFTER TRYPTOPHANE ADMINISTRATION IN RELATIONSHIP TO THE OCCURRENCE OF SYMPTOMS AND SIGNS OF PYRIDOXINE DEFICIENCY IN PATIENTS RECEIVING DESOXYPYRIDOXINE

PATIENT	XANTHURENIC ACID LEVEL (MG./24 HR.)			MAJOR SIGNS OF PYRIDOXINE LACK	OCCURRENCE OF XANTHURENIC ACID IN RELATION TO MAJOR SIGNS
	BEFORE DESOXY-PYRIDOXINE	WITH DESOXY-PYRIDOXINE	AFTER PYRIDOXINE		
F. B.	12	56	13	0	
Z. L.	21	119	–	0	
C. N.	3	76	–	(glossitis dermatitis)	concurrent
J. B.	13	271	17	0	
C. G.	24	247	7	0	
J. M.	20	243	29	0	
J. C.	12	23	–	0	
H. M.	16	172	17	dermatitis	before
W. W.	24	179	–	0	
J. D.	14	197	–	0	
D. B.	20	152	–	0	
C. McM.	57	45	–	0	
M. B.	7	303	–	(glossitis dermatitis)	before
L. D.	31	245	10	(glossitis dermatitis)	after
G. W.	90	508	16	dermatitis	before
J. M.	8	139	7	dermatitis	after
K. W.	16	149	–	0	
J. W.	0	221	–	dermatitis	before
D. F.	14	166	–	dermatitis	after
F. L.	0	314	–	dermatitis	before
M. K.	14	214	3	dermatitis	before
E. J.	65	500	70	glossitis	before
D. E.	47	293	25	dermatitis	before
J. M.	60	249	36	dermatitis	before
M. L.	59	108	10	(glossitis dermatitis)	before
L. B.	0	28	–	0	
J. P.	15	137	31	0	
H. P.	16	67	7.5	(dermatitis cheilosis)	concurrent
F. C.	37	85	25	dermatitis	before
F. C.	47	139	80	dermatitis	before
R. P.	20	96	23	0	
R. P.	38	171	41	dermatitis	before
C. P.	38	239	34	dermatitis	concurrent
F. D.	35	85	22	(dermatitis glossitis neuritis)	after

the amount of this metabolite excreted in the urine could be found when a period before the administration of desoxypyridoxine was compared with the deficiency period or the period after the administration of pyridoxine.

When vitamin free casein,* 100 Gm. per day, was added to the B-complex-poor diet of four patients during the deficiency period, no changes were noted in the amounts of xanthurenic acid or of N′-methylnicotinamide that were excreted after a tryptophane load test. These patients did not develop deficiency manifestations more rapidly or in a more florid form.

The metabolism of other nitrogenous substances, glucose and cholesterol: Blood urea nitrogen levels tended to rise during the deficiency period. (See Table III.) Urine urea nitrogen levels were unchanged. No changes were noted in blood and urine uric acid levels, or in serum proteins.

Blood urea nitrogen levels were determined in three subjects before and twelve hours after the administration of 30 Gm. of alanine. In the control period, the urea nitrogen level after the load test was the same or lower than the level before the load test. After lesions of vitamin B_6 deficiency appeared in two patients, the urea nitrogen levels rose above normal, and the level after the load test rose even higher. After pyridoxine was administered, the urea nitrogen levels decreased to the control range. The third patient (J. B.) did not develop lesions of vitamin B_6 deficiency, and no changes of significance were noted in the blood urea nitrogen levels. (See Fig. 9.) The high initial level in J. M. can be explained by the much larger protein intake on the regular hospital diet. This decreased as soon as he was placed on the 30 Gm. protein vitamin-B-complex-poor diet, but the urea nitrogen subsequently rose as the deficiency state progressed.

Blood glucose values determined with the patient fasting were within the normal range before, during, and after the deficiency state. The administration of alanine did not affect the blood glucose levels determined twelve hours later. The results of oral and intravenous glucose tolerance tests were not altered by the deficiency state, nor were blood lipids, blood cholesterol, or cholesterol esters. Significant changes were not observed in the blood level of cholesterol or its esters determined before and six and twenty-four hours after a high-fat, high-cholesterol meal.

The function of the adrenal cortex: No significant changes were noted in the following determinations before and during the deficiency state and after

TABLE III. UREA NITROGEN IN MILLIGRAM/PER CENT; SUBJECT FASTING

	BEFORE DESOXYPYRIDOXINE	AT HEIGHT OF PYRIDOXINE DEFICIENCY	AFTER PYRIDOXINE
H. P.	11	19	12
M. L.	9	13	8
J. M.	11	20	9
D. E.	16	21	18
J. B.	15	20	19

The effect of pyridoxine deficiency upon the blood urea nitrogen taken with the patients fasting. Another patient on the same diet and in the same institution received saline injections instead of desoxypyridoxine. At comparable periods, his blood urea nitrogen levels were 12 mg. per cent; 12 mg. per cent; 11 mg. per cent.

*Nutritional Biochemical.

pyridoxine was added to the regime: serum sodium, potassium and 17 ketosteroid levels, the Kepler-Power water test, and the response of eosinophils, polymorphonuclear neutrophils, and lymphocytes to the administration of 40 mg. of ACTH intramuscularly. No significant variations from the normal were

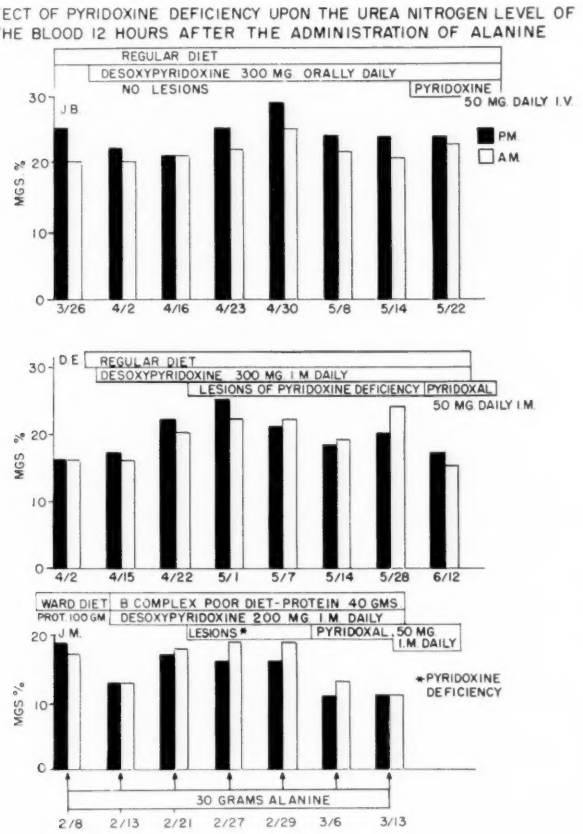
EFFECT OF PYRIDOXINE DEFICIENCY UPON THE UREA NITROGEN LEVEL OF THE BLOOD 12 HOURS AFTER THE ADMINISTRATION OF ALANINE
REGULAR DIET
DESOXYPYRIDOXINE 300 MG. ORALLY DAILY
NO LESIONS
PYRIDOXINE 50 MG. DAILY I.V.
J.B.
PM.
A.M.
MGS %
3/26 4/2 4/16 4/23 4/30 5/8 5/14 5/22
D.E.
REGULAR DIET
DESOXYPYRIDOXINE 300 MG. I.M. DAILY
LESIONS OF PYRIDOXINE DEFICIENCY
PYRIDOXAL 50 MG. DAILY I.M.
4/2 4/15 4/22 5/1 5/7 5/14 5/28 6/12
WARD DIET
PROT 100 GM
B COMPLEX POOR DIET-PROTEIN 40 GMS
DESOXYPYRIDOXINE 200 MG. I.M. DAILY
J.M.
LESIONS*
PYRIDOXAL, 50 MG. I.M. DAILY
*PYRIDOXINE DEFICIENCY
30 GRAMS ALANINE
2/8 2/13 2/21 2/27 2/29 3/6 3/13

Fig. 9.—This chart demonstrates the effect of pyridoxine deficiency induced by desoxypyridoxine on the deamination of a test dose of alanine. J. B. who developed no lesions had repeated normal responses to the alanine, i.e., blood urea nitogen levels had returned to normal in twelve hours. D. E., and J. M. had higher blood urea nitrogen levels twelve hours after the test dose of alanine than just before the alanine was administered. This effect was observed during the period of clinical pyridoxine deficiency, whereas the response was normal before the pyridoxine deficiency state and after pyridoxine was administered.

noted in any of these tests which could be ascribed to vitamin B_6 deficiency. Even though lymphopenia and polymorphonuclear neutrophil leukocytosis had occurred as a result of the deficiency, further decrease in numbers of lymphocytes and an additional increase in numbers of polymorphonuclear neutrophilic leukocytes occurred in response to ACTH. (See Fig. 10.)

DISCUSSION

Vitamin B_6 deficiency has been induced in human beings by a pyridoxine antagonist, desoxypyridoxine in conjunction with either a diet poor in the

vitamins of the B complex or a regular hospital diet. The clinical signs that develop are those that usually are found in other vitamin B complex deficiency states. The seborrheic dermatitis beginning in the nasolabial folds and spreading laterally over the face and sometimes involving the buttocks, scrotum, and

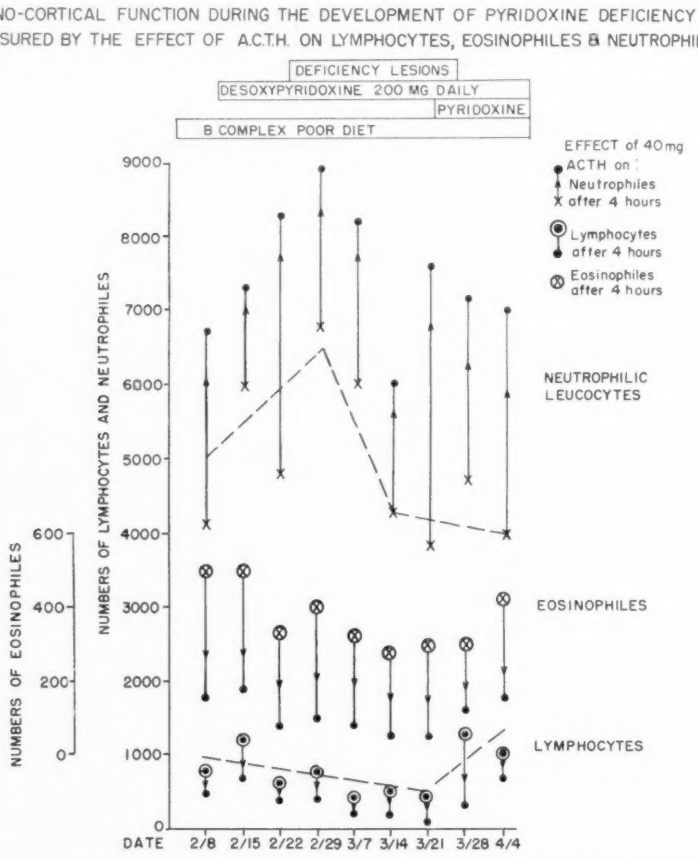

Fig. 10.—This chart demonstrates that pyridoxine deficiency induced by desoxypyridoxine does not interfere with the lymphopenic,eosinopenic, and neutrophilic effect of adrenal stimulation by ACTH.

perineum has been described as a manifestation of riboflavin deficiency by Jolliffe[44] and has been induced in volunteers on a riboflavin deficient diet by Horwitt[45, 46] at the Elgin State Hospital. Facial lesions are more common in the patients with pyridoxine deficiency. Scrotal lesions are more common in the riboflavin deficient group. Cheilosis, angular stomatitis, and conjunctivitis similar to the signs of riboflavin deficiency have also occurred in the vitamin B_6 deficient subjects. None of these abnormalities have responded to riboflavin, but heal quickly when pyridoxine is added to the experimental regime.

Glossitis, stomatitis, and dermatitis have occurred which are similar to the lesions of tongue, mouth, and skin seen in natural and experimentally in-

duced niacin deficiency.[47] These lesions have not responded to niacin but disappear rapidly when pyridoxine is administered. Cozymase, a niacin containing pyridine nucleotide, also has been effective in eliminating the lesions of tongue and mouth.

Severe neuropathy has occurred which has involved sensory nerves primarily and has simulated thiamin deficiency neuritis. There has been no response to thiamin, but pyridoxine has eliminated these symptoms and signs.

There is nothing specific about these vitamin deficiency signs. Vitamin B_6 deficiency has mimicked them all, and it is quite likely that this mimicry is one reason why vitamin B_6 deficiency has not been recognized as a deficiency disease of human beings occurring under natural conditions of dietary inadequacy.

It is possible also that vitamin B_6 deficiency interferes with the formation of some of the coenzymes, of which the B complex vitamins are the precursors. This possibility seems likely because cozymase induces a remission in vitamin B_6 deficiency glossitis and stomatitis, though niacinamide does not. There has not been opportunity as yet to test the other coenzymes, thiamin pyrophosphate, and flavin adenine nucleotide against the neuritis and the cheilosis and conjunctivitis, respectively.

In rats, dietary vitamin B_6 deficiency accentuated by desoxypyridoxine interferes with the conversion of tryptophane to liver pyridine nucleotides,[13] and to an excretory metabolite, N′-methylnicotinamide.[9, 10] Xanthurenic acid is an abnormal metabolite of tryptophane excreted in the urine as the result of a side reaction when the main steps in this conversion through kynurenine, 3-hydroxykinurenine, 3-hydroxy-anthranilic acid, and quinolinic acid are impeded.[48] Since xanthurenic acid occurs in the urine of vitamin B_6 deficient patients given test doses of tryptophane, it seems likely that vitamin B_6 deficiency interferes with normal tryptophane metabolism in human beings also. In these studies, however, there was no evidence for interference with the conversion of tryptophane to N′-methylnicotinamide. Snyderman, Carretero, and Holt[2] have reported that such a metabolic abnormality can be demonstrated in pyridoxine deficient infants. Elvehjem[49] has suggested that the difficulties encountered in demonstrating an abnormality in this reaction even in some vitamin B_6 deficient animals depends upon the degree to which tryptophane is converted to pyridine coenzymes in the various species. In adult persons the magnitude of this conversion is small (10 Gm. *dl* tryptophane induce an increment of only 5 to 10 mg. per 24 hours of N′-methylnicotinamide in the urine) and the ease with which interference can be demonstrated is reduced proportionally. Perhaps if tryptophane were fed at the 10 Gm. level daily, a metabolic abnormality could be demonstrated. The suggestion has been made also that the amount of protein available to pyridoxine deficient animals influences the conversion. In experiments with rats, high protein interfered and low protein facilitated the reaction.[50] No such relationship could be demonstrated in our deficient subjects whose diets were supplemented with 100 Gm. of vitamin free casein, even though one would anticipate that extra purified protein in the diet would increase the vitamin B_6 requirements.

Vitamin B_6 functions also in transamination and decarboxylation reactions, and through them is of theoretical importance in protein and amino acid metabolism. Indirectly through the release of amino groups, it effects the formation of urea. If an amino acid like alanine is administered to a person deficient in vitamin B_6, deamination with subsequent urea formation should be delayed and the urea should be elevated in the blood for a longer time than in the normally nourished person. An alanine test of this type has been proposed and has been investigated in vitamin B_6 deficient rats and in pregnant women.[25] Trials of this test in three of our vitamin B_6 deficient patients indicates that the blood urea nitrogen does tend to remain elevated twelve hours or more after the test dose of alanine, whereas before the deficiency state and after treatment with pyridoxine the urea nitrogen levels have returned to normal in twelve hours. The magnitude of the change is not great but is the same as that recorded in women with hyperemesis gravidarum who received alanine.[25] This metabolic abnormality probably accounts for the general tendency of the blood urea nitrogen to rise as the vitamin B_6 deficiency state increased in severity. The rise noted in our deficient patients is comparable to the rise noted in vitamin B_6 deficient rats.[51] By contrast, women with hyperemesis gravidarum had low blood urea levels initially probably because of failure to retain food. These levels rose after the administration of pyridoxine. The dependence of protein metabolism upon the vitamin B_6 group suggested that a high protein diet might increase demands for vitamin B_6. However, when the protein content of the diet of vitamin B_6 deficient patients was increased by 100 Gm. of vitamin free casein the severity of the signs and symptoms of vitamin B_6 deficiency were not altered. None of these experiments were continued for more than three weeks which may have been insufficient time to demonstrate an intensification in the deficiency state.

In rats, vitamin B_6 deficiency and essential fatty acid deficiency induce a similar type of dermatitis.[11, 12] When both deficiency states are present in the same animal, both essential nutrients are necessary to heal the lesion. However, when vitamin B_6 deficient rats are treated with large amounts of linoleic acid, the skin lesions appear more slowly and are less severe. There appears to be a relationship between pyridoxine and essential fatty acid metabolism. In addition, chronic pyridoxine deficiency induces arteriosclerotic lesions in monkeys.[52] These observations prompted tests for lipid and cholesterol in the blood of the pyridoxine deficient patients as well as the therapeutic tests with linoleic acid. No gross changes in lipid blood constituents were found but skin lesions improved in two of the four patients who received the largest dose of linoleic acid for four weeks or more. These therapeutic observations support the concept of a metabolic relationship of the vitamin B_6 group to the essential fatty acids.

The lymphocytopenia and polymorphonuclear leukocytosis observed in these pyridoxine deficient patients suggested the possibility that these effects were dependent upon increased adrenal cortical activity or that cortical steroids induced similar hematologic changes by interfering with vitamin B_6 metabolism. Hyperplasia of the adrenal glands in vitamin B_6 deficient

monkeys[53] and rats [54, 55] added additional evidence for such a relationship, though the evidence from the animal work favored depletion rather than hyperfunction of the adrenals. However, studies on adrenal cortical function in these patients gave no evidence for hyper- or hypofunction. The vitamin B_6 deficiency effect was accentuated by ACTH, and pyridoxine in 100 mg. doses daily for one week did not inhibit the lymphopenia or neutrophilic leukocytosis of ACTH in two normal subjects. Recently Mueller, Weir, and Heinle[56] have shown that vitamin B_6 deficiency lymphocytopenia and polymorphonuclear neutrophil leukocytosis occur in adrenalectomized rats, and have reported results which are essentially the same as those observed in our patients.

These studies do indicate that vitamin B_6 is an essential nutrient for human beings and that the signs of vitamin B_6 deficiency are the same as the signs of other vitamin B complex deficiency diseases. In areas where mixed vitamin B complex deficiency states are endemic, the extent of pyridoxine deficiency can be established only by chemical tests for (1) xanthurenic acid in the urine after test doses of tryptophane, (2) urea nitrogen in the blood before and after test doses of alanine, (3) the vitamin B_6 group in blood and tissues, and (4) therapeutic tests with pyridoxine on the relief of symptoms, signs, and altered biochemistry.

These studies do not pinpoint the vitamin B_6 requirement for human beings. However, since 5 mg. of pyridoxine is sufficient to heal lesions of vitamin B_6 deficiency induced by 200 mg. of desoxypyridoxine in a patient whose diet contains approximately ½ mg. of pyridoxine, one may deduce that more than ½ mg. and less than 5 mg. will meet requirements of adult human beings very satisfactorily. This checks well with the figures of Register, Lewis, Ruegamer, and Elvehjem.[57] It is clear, however, that very severe restriction is required before symptoms of the deficiency state will appear.

SUMMARY

Clinical signs of vitamin B_6 deficiency have been induced in 34 of 50 patients who received desoxypyridoxine while consuming an adequate diet or one deficient in the vitamins of the B complex.

These signs are (a) anorexia, nausea, listlessness, and lethargy; (b) seborrhea sicca-like dermatitis; (c) cheilosis and conjunctivitis; (d) glossitis; (e) pellagra-like dermatitis; and (f) polyneuritis. Vitamin B_6 deficiency mimics the other B complex deficiency diseases.

Lymphocytopenia occurred in the peripheral blood. Eosinophilia was noted when skin lesions were extensive. Mild anemia which occurred in five patients could not be related directly to the vitamin B_6 deficiency state. No changes occurred in the bone marrow.

Abnormalities were demonstrated in the metabolism of tryptophane, alanine and urea.

Cozymase relieved glossitis after nicotinamide had failed, suggesting that there may be an abnormality in the formation of the pyridine coenzymes provoked by vitamin B_6 deficiency.

A direct effect of vitamin B_6 deficiency on fat metabolism or adrenal cortical activity could not be demonstrated. The effects of linoleic acid on pyridoxine deficiency dermatitis suggests that a metabolic relationship exists between the essential fatty acids and the B_6 group of vitamins.

Pyridoxine is absorbed and metabolized by the skin. If applied locally in small amounts it will heal the skin lesion at the site of application. If applied over a larger skin area it will relieve all clinical and chemical signs of vitamin B_6 deficiency.

Pyridoxine, pyridoxal, or pyridoxamine given orally, parenterally, or in an ointment on the skin will alleviate the clinical and chemical manifestations of vitamin B_6 deficiency, though the patient remains on the same regime that induced the deficiency signs.

Pyridoxine is an essential nutrient for human beings. Two to three milligrams will meet the daily requirements of adult human beings very satisfactorily.

The following case report will indicate how the major lesions of pyridoxine deficiency appear, progress and involute in response to pyridoxine or other agents.

CASE 1.—F. D., a 54-year-old white man with chronic rheumatoid arthritis, was continued on his customary low-sodium, high-protein diet supplemented with a mixed vitamin capsule containing thiamin, niacin, and riboflavin twice a day. On July 21, 1952, he was started on desoxypyridoxine 150 mg. twice daily. He excreted 35 mg. of xanthurenic acid in the urine after 10 Gm. of *dl* tryptophane. The dose of desoxypyridoxine was increased to 200 mg. twice daily on July 30. On August 10 he noted itching in the nasolabial folds. The next day erythema and flaky dermatitis were observed in these areas. The lips were fissured and the corners of the mouth were macerated. By August 15 there were oily yellowish scales on a mildly reddened base in the eyebrows, on the scalp, and behind the ears. The amount of xanthurenic acid in the urine had not increased. On August 25 a similar skin lesion appeared on the scrotum extending over the perineum. Linobione (linoleic acid) 5 Gm. daily in divided doses was added to the experimental regime. This dose was increased to 8 Gm. daily on September 8 inasmuch as no change had been noted in the skin lesions or lips. On September 10 glossitis and stomatitis appeared. The papillae at the tip of the tongue appeared as small red dots on a smooth swollen background. There were superficial ulcerations surrounded by red areolae on the oral mucous membrane. The mouth and tongue were sore, and the patient was unable to take hot food or drinks. By September 12 the pharynx had become orange-red and sore, but the skin lesions had begun to improve, erythema had subsided, and there was less scaling. Xanthurenic acid output had increased to 85 mg. per twenty-four hours. By September 25 the skin had cleared except for slight residual scaling, but the oral lesions were static. On this day 25 mg. of cozymase was given intravenously by slow drip. The next day the patient could swallow hot coffee, and the oral mucous membranes were less reddened. The cozymase was readministered on six succeeding days, and by September 27 all evidences of glossitis and stomatitis had vanished, and the output of xanthurenic acid was 22 mg. per twenty-four hours. Normal filiform papillae were visible on the tip of the tongue, and all oral symptoms had disappeared. By October 2 there had been no recurrence of the glossitis, so cozymase was discontinued. There was little or no seborrhea evident, but the patient began to complain of numbness and tingling sensations in the feet. Hyperesthesia to pin and light touch extended up to the ankles and vibration perception, previously present in the ankles and knees, was absent. Perception of position was intact, but the ankle jerks were absent and the knee jerks reduced in activity. By October 3 the neuritis was progressing rapidly. Hyperesthesia and afterglow extended 6 inches above the ankles, and there was

numbness and tingling in the fingers. The sciatic nerve trunks were tender and the patient was unable to sleep or continue his physiotherapy because of pain and burning. The neuritis continued to progress as indicated by the rising level of hyperesthesia. Discomfort, weakness, and anorexia were pronounced. On October 6, 50 mg. of pyridoxine was given and repeated daily. Thereafter there was slow regression of the neuritis, and with it return of appetite and strength. By October 16 the level of hyperesthesia was at the ankles. By October 19 all subjective and objective changes in the fingers had disappeared, and the tuning fork was perceived at the ankles. By November 5 it was recognized in the toes. The nerve trunks were no longer tender, and the ankle jerks were present but hypoactive. Subjective burning of the soles of the feet with electric shocks shooting up the legs continued long after all objective evidences of neuritis had disappeared and were still present but much less severe on Jan. 24, 1953, when desoxypyridoxine and pyridoxine were discontinued.

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RADIOACTIVE SODIUM CHROMATE FOR THE STUDY OF SURVIVAL OF RED BLOOD CELLS

I. The Effect of Radioactive Sodium Chromate on Red Cells

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THE usefulness of radioactive sodium chromate ($NaCr^{51}O_4$) as a tracer substance for the measurement of blood volume was reported by Gray and Sterling in 1950.[1, 2] Their data suggested that the radiochromate method could not be employed to study red-cell survival time since the Cr^{51} disappeared from the circulation more rapidly than could be explained on the basis of the known rate of destruction of red cells as demonstrated by the Ashby method,[3] or by isotopic technique using N^{15}, Fe^{59}, or C^{14}.[4-6] Since the curve for the disappearance of Cr^{51} from the blood is approximately linear with respect to time, it occurred to us that the chromium-labeled red cells could be used to make relative estimates of the survival of erythrocytes under clinical conditions. Because of the simplicity and the versatility of the radiochromium technique, it seemed desirable to explore this possibility even though it was evident that absolute survival time could not be determined directly. In this paper we report preliminary studies that were undertaken to investigate: (1) the nature of the combination of $NaCr^{51}O_4$ with the red cell; (2) the effect of the chromate on the cell; (3) the rate of elution of Cr^{51} from the cells; and (4) the normal apparent survival curves.

METHODS AND RESULTS

Whole blood, either heparinized or in ACD solution, was diluted with one-half volume of physiologic solution of sodium chloride. To this was added radioactive sodium chromate* in sufficient amount to give approximately 200 μc per 100 ml. of blood. The mixture was allowed to stand at room temperature for one hour with frequent agitation. The blood was then centrifuged in the cold for fifteen minutes at 2,000 r.p.m. and the supernatant removed. The red cells were washed three times in this manner, and then resuspended in cold salt solution. An aliquot was taken for the preparation of a standard, and the remainder was either reinjected or used for in vitro experiments. The effect of the anticoagulant used is shown in Table I.

It is evident that somewhat better labeling is obtained using ACD, although the difference is not sufficiently great to interfere with our studies. The radiation measurements were made using either the Texas-type well counter, or a well-type crystal counter. With

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*Procured from the Radioactive Pharmaceuticals Division of the Abbott Laboratories under allocation from the Isotopes Division, U. S. Atomic Energy Commission. It is supplied as a solution of $NaCr^{51}O_4$ in benzoyl alcohol buffered with sodium acetate-acetic acid to pH 7.0. The specific activity on delivery has varied from 240 to 1100 mc/g. The chromium used in labeling has ranged from 2.5 to 7.5 micrograms per ml. of whole blood. The total amount of radioactivity injected has varied from 100 to 150 μc. According to Handbook 52 of the National Bureau of Standards, the maximum permissible internal dose of Cr^{51} in the total body is 390 μc. (Table C, p. 15.)

TABLE I. THE EFFECT OF THE ANTICOAGULANT ON THE UPTAKE OF RADIOACTIVE SODIUM CHROMATE

METHOD A: WHOLE BLOOD DILUTED WITH ONE-HALF VOLUME OF SALT SOLUTION BEFORE ADDITION OF Cr^{51}

METHOD B: PLASMA REPLACED BY EQUAL VOLUME OF SALT SOLUTION BEFORE ADDITION OF Cr^{51}

METHOD	ANTICOAGULANT	ACTIVITY OF REACTION MIXTURE NET C/M/ML	ACTIVITY OF SUPERNATANT NET C/M/ML	PER CENT UPTAKE OF Cr^{51}
A	Heparin	2,644	925	67
	ACD	2,531	354	86
B	Heparin	2,431	887	64
	ACD	2,398	394	84

these devices it is possible to use samples of whole blood, or other liquid, in volumes of 1.0 to 4.0 ml. The efficiency of our Texas-type counter for the gamma radiation of Cr^{51} is approximately 0.3 per cent, and for the crystal scintillator it is approximately 3.0 per cent. In all experiments the net counts per minutes per unit of sample are corrected for decay to zero time.

(1) *The Nature of the Combination of $NaCr^{51}O_4$ With the Red Cell.*—Labeled human erythrocytes, or labeled sheep erythrocytes, in 50 per cent suspension, were hemolyzed by the addition of 10 volumes of distilled water. The stroma was separated by repeated filtration through a Seitz filter. The stroma was then washed three times with distilled water. The distribution of the radioactivity between the washed stroma and hemoglobin is shown in Table II.

TABLE II. DISTRIBUTION OF RADIOACTIVITY BETWEEN STROMA AND HEMOGLOBIN

	NET C/M/ML	PER CENT OF TOTAL*
Suspension of red cells	18,013	100
Washed stroma	3,996	22
Hemoglobin solution	14,017	78

*Average of three experiments.

The per cent of radioactivity in the hemoglobin solution is somewhat less than the 97 per cent reported by Gray and Sterling. There is no apparent explanation for this difference. Concentrated hydrochloric acid was then added to the hemoglobin solution to separate the globin hydrochloride from the hematin. The globin fraction contained 70 per cent of the radioactivity of the original solution. This compares well with the value of 78 per cent reported by Gray and Sterling. The hematin was found to contain an insignificant amount of radioactivity.

(2) *The Effect of the Chromate on the Red Cell.*—The effect of sodium chromate on the mechanical fragility of human and sheep erythrocytes was studied in the following manner. Red cells in ACD solution were labeled as described above. Unlabeled red cells, treated in an otherwise identical fashion, served as a control. Sample I was treated with the usual amount of $NaCr^{51}O_4$: 2 μc per milliliter of fresh blood, before washing. The specific activity was such that the amount of chromium metal added was 30 micrograms per milliliter. Sample II was treated with twice this amount of radioactive chromium. Sample III was treated with 30 micrograms per milliliter of "cold" $NaCr^{51}O_4$

solution, and Sample IV, with twice this amount, or 60 micrograms per milliliter. The tubes containing the labeled blood and the control blood were agitated constantly at 20° C. for the duration of the experiments. Aliquots were removed from each sample daily and analyzed for total hemoglobin, and hemoglobin in the supernatant, by the method of Bing and Barker.[7] The percentage of hemoglobin in the supernatant indicated the extent of hemolysis. The results are shown graphically in Fig. 1. It can be seen (Samples I and III) that the amount of Cr^{51} ordinarily used, and as much as ten times the amount of chromium metal ordinarily used, has no effect on the mechanical fragility of the washed red cells. When the amount of chromium ordinarily used is increased by a factor of 20 there is a significant increase in mechanical fragility. Doubling the amount of radioactivity, however, has no influence on fragility.

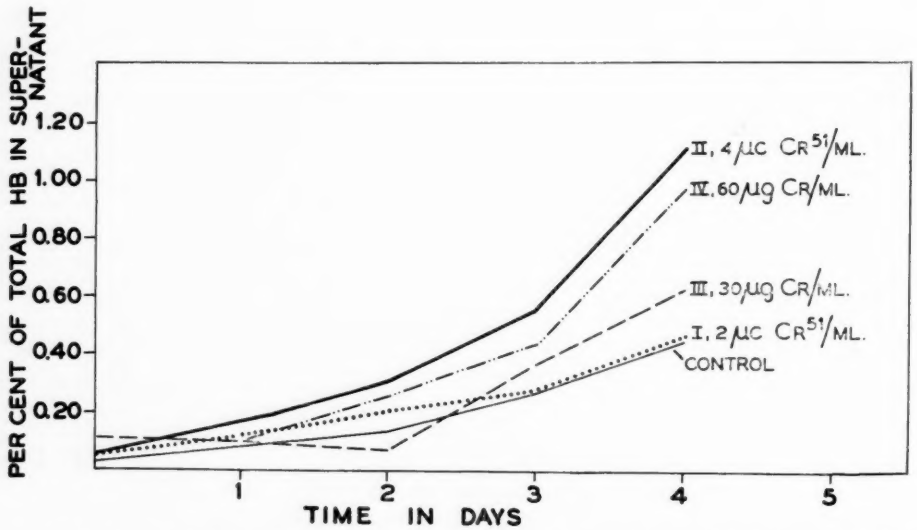

Fig. 1.—The effect of sodium chromate on hemolysis in vitro. Whole blood was treated either with radioactive sodium chromate or "cold" sodium chromate. The amounts of chromate added to the whole blood before washing are shown on the figure. The specific activity of the radioactive sodium chromate solution was such that 2 microcuries of Cr^{51} per milliliter contained 30 μg of metallic chromium per milliliter. The five curves shown on the graph were obtained with aliquots of the same sample of blood.

(3) *The Rate of Elution of Cr^{51} From Red Cells.*—Labeled washed human red cells in a 50 per cent suspension were dialyzed for fourteen days against physiologic solution of sodium chloride. Two milliliters of the red cell suspension were placed in a Visking tube which was agitated in 40 ml. of dialysate at 20° C. The tubes containing the red cells and the dialysate were counted daily in the Texas well-type counter. The results of a typical experiment are shown in Table III. The elution of Cr^{51} from the labeled cells can be expressed by the equation:

$$\log X_t = \log X_o - Kt,$$

where $\log X_o$ = per cent of radioactivity in the cells at day 0, = 2.000 (100

per cent), and log X_t = per cent of radioactivity in the cells at day t, and K = a constant.

The values for K on representative days are shown in Table III. The average over a period of fourteen days (excluding the first day) is 0.0038, which amounts to a loss of 1.009 per cent of the remaining radioactivity per day. At this rate the half-time for the elution of Cr^{51} would be seventy-five to eighty days. This value is in good agreement with that reported by Ebaugh, Ross, and Emerson.[8]

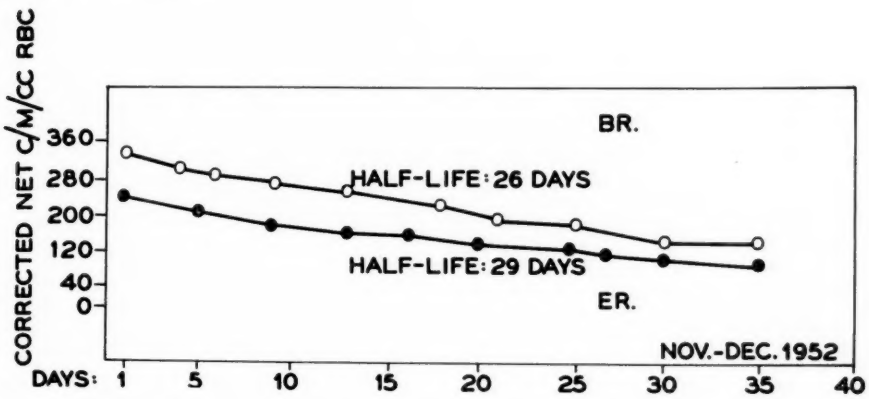

Fig. 2.—Typical curves for apparent survival time of group O red cells administered to group A recipients. The recipients were normal volunteers and the group O blood was obtained and labeled with Cr^{51} on the same day. The intervals of the ordinate are the same for all figures.

(4) *The Normal Apparent Survival Curves.*—The Cr^{51}-labeling technique was studied in 14 normal subjects using three experimental conditions: (a) Labeled group O blood in group A recipients. (b) Labeled genotype-specific blood in compatible recipients. (c) The individual's own cells labeled and reinjected.

After injection of Cr^{51}-labeled red cells, samples of heparinized blood were obtained at frequent intervals, and the hematocrit determined. Aliquots of 3.0 ml. were taken using siliconed pipettes. Each aliquot was counted twice in the well-type counter. Sufficient total counts were accumulated so that the probable error of counting was less than 5 per cent. A standard sample was also counted twice to correct for decay, geometry, and counter efficiency. The standard consisted of 3.0 ml. of a 1:30 dilution, in blood, of the labeled cells injected. The net counts per minute per milliliter of the sample corrected for the hematocrit was divided by the net counts per minute per milliliter of the standard. The results were plotted on the ordinates of semilogarithmic paper against time in days.*

Fig. 2 shows representative apparent survival curves obtained using group O labeled blood in group A recipients. The donor blood was collected in ACD

*In the case of the first group of normal subjects, the actual net counts per minute per milliliter were derived from the counting data. The use of the standard sample and the technique of reporting ratios of net counts per minute was developed to expedite the handling of the data.

solution, and was less than twenty-four hours old. The apparent half-survival time in three subjects under these conditions was found to be twenty-nine, twenty-six, and twenty-five days, respectively.

Fig. 3 shows typical apparent survival curves obtained using labeled genotype-specific blood injected into compatible recipients. The blood was drawn into a solution of heparin in preparation for the Cr^{51} labeling. Five such experiments have been performed. The apparent half-survival time under these conditions was found to be thirty-nine, thirty-five, thirty-four, thirty-eight, and thirty days, respectively.

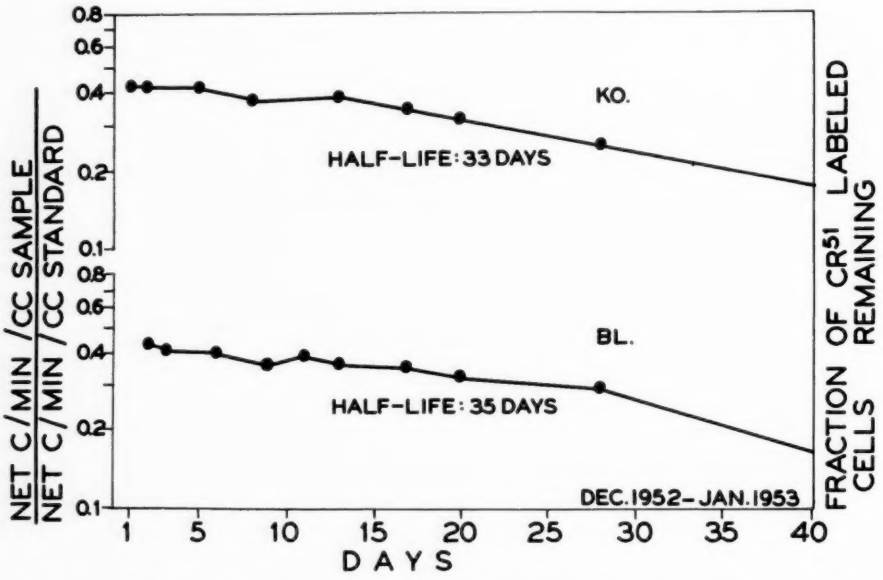

Fig. 3.—Typical curves for apparent half-survival time of genotype-specific blood in compatible recipients. The donor blood was labeled with Cr^{51} immediately after it was withdrawn; and the washed labeled cells were reinjected as soon as possible.

Fig. 4 shows the typical curves obtained when the individual's own cells were labeled and reinjected. The blood for these experiments was drawn into a solution of heparin in preparation for the Cr^{51} labeling. Five such experiments have been performed. The apparent half-survival times under these conditions were found to be thirty, thirty-three, thirty, thirty, and thirty-three days, respectively.

DISCUSSION

The experimental data presented in this report demonstrate that the rate of disappearance of Cr^{51} from the blood of normal subjects is a function of time. The time required for the disappearance of half of the radioactivity displays individual variations, but is of the order of thirty to forty days. This time is significantly shorter than the values obtained using the Ashby, or other isotope techniques where one measures the actual rate of disappearance of

individual cells representative of a mixed population which is assumed to have a normal age distribution. Using the latter techniques, the half-disappearance time also displays individual variations, and is of the order of fifty-five to seventy days. Since it has been shown that there is elution of Cr^{51} from the red cells, it is not surprising to find that the disappearance curve in this case is exponential. For these reasons we have chosen to use the phrases "apparent survival time," and "apparent half-survival time" to describe the results of our studies with radiochromium.

Since all three techniques measure a change in the blood as a function of time, it is of interest to examine the relationship between the curves of actual

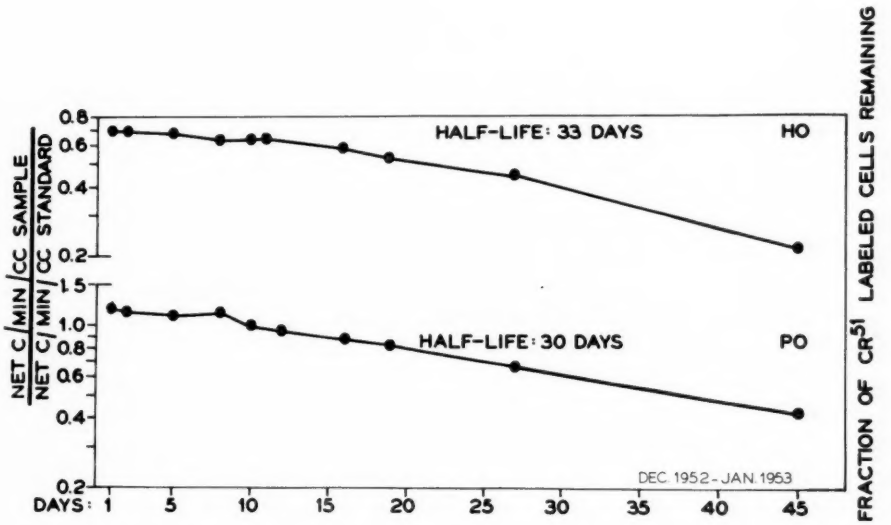

Fig. 4.—Typical curves of the apparent half-survival time of the individual's own red cells labeled with Cr^{51} and reinjected.

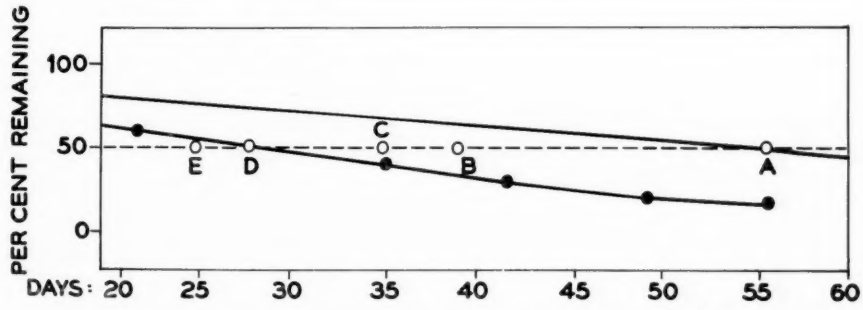

Fig. 5.—The influence of the rate of elution of Cr^{51} on the apparent half-survival time of red cells. The upper curve crosses the 50 per cent ordinate at "A" or approximately fifty-five days. This is a typical curve of red-cell survival obtained by the Ashby technique using group O blood in a recipient of group A. The lower curve has been constructed by subtracting approximately 7.5 per cent per week (1+ per cent per day) from the Ashby curve. This curve crosses the 50 per cent ordinate at approximately twenty-eight days, ("D"). The points on the 50 per cent ordinate marked *B*, *C*, and *E* are typical values for the apparent half-survival time of red cells determined by the radiochromate method.

TABLE III. THE ELUTION OF Cr^{51} FROM LABELED RED CELLS

DAY	TOTAL Cr^{51} BOUND TO RED CELLS		TOTAL Cr^{51} IN DIALYSATE†		K‡
	NET C/M*	PER CENT	NET C/M	PER CENT	
0	8496	99.2	63	0.7	
1	8024	98.6	121	1.5	0.0061
2	7977	98.2	142	1.8	0.0039
3	7735	97.7	242	2.3	0.0033
4	7416	95.8	318	4.2	0.0048
8	7134	93.0	521	7.0	0.0040
12	7110	91.2	690	8.8	0.0033
14	7217	87.2	1063	12.8	0.0043

*All counts corrected for decay to zero time. No correction for counter efficiency.
†Cr^{51} in dialysate corrected to include Cr^{51} eluted from cells and within the Visking tube.
‡See Equation 1.

and apparent survival time. In the study of the rate of elution of Cr^{51} from red cells, it was shown that the loss of remaining radioactivity per day was approximately 1+ per cent. At this rate, the half time for the elution of Cr^{51} is seventy-five to eighty days. If a typical curve of an Ashby-type experiment is corrected by the loss of approximately 7+ per cent per week, the actual half-survival time of fifty-five days is reduced to thirty days, and the linear curve becomes exponential. This operation is illustrated in Fig. 5. The Ashby curve *(A)* was obtained in the usual manner, employing fresh group O blood in a recipient of group A. The corrected curve passes through point D, which is the apparent half-survival time obtained in one of our normal controls: curve *E* of Fig. 2. In this case group O blood, labeled with Cr^{51}, was given to a recipient of group A. The other points on the 50 per cent ordinate indicate the apparent half-survival times of the other normal subjects described above. It appears quite evident to us that the Cr^{51}-labeling technique measures the actual survival time of red cells corrected for the rate of elution of the radioactive material.

In spite of this clear relationship, we prefer to use the expression "apparent survival time" since we have no proof that the rate of elution of Cr^{51} is the same in vivo as in vitro, nor have we evidence that the rate of elution is the same from normal and abnormal cells. It seems probable to us that the loss of Cr^{51} from its globin binding is a physical-chemical phenomenon, which is independent of the experimental conditions mentioned. Since we are interested in the development of a technique to follow the course of hemolytic processes, we prefer not to apply an arbitrary correction factor for elution. We see no objection to the use of the expression "apparent half-survival time" as a measure of relative rate of destruction of blood. In the case of normal individuals, the apparent half-time (thirty to forty days) is sufficiently long for most purposes; and in the case of abnormal individuals the marked reduction of the half-time minimizes the discrepancy between the methods. In cases where serial studies are made it is evident that the results of before-and-after studies can be expressed as ratios, which should be comparable for the various methods.

It is proper to speculate on the influence of the sodium chromate used for tagging on the resistance of red cells to the normal process of blood destruc-

tion. The evidence available suggests that neither the amount of chromate used nor the radiations emitted have any influence on the susceptibility to destruction of the labeled cells. It was obviously difficult to design an experiment in vivo which would be conclusive, but the indirect evidence seems adequate. It was shown in Fig. 1 that excessive amounts of chromate ion increased the mechanical fragility of erythrocytes, whereas the beta and gamma radiation apparently had no effect. When blood is labeled according to our methods, the concentration of chromium metal has ranged from 2.5 to 7.5 micrograms per milliliter of whole blood, depending upon the specific activity of the $NaCr^{51}O_4$ solution used. It is evident from the graph that as much as 30 micrograms of chromium per milliliter had no significant effect on fragility. However, when the amount of chromium metal was increased to 60 micrograms per milliliter, there was a definite increase in mechanical fragility. On this basis, it is preferable to use $NaCr^{51}O_4$ of high specific activity; and batches should not be used if the dose of chromium per milliliter of whole blood will exceed 30 micrograms. The other evidence for the lack of harmful effect from acceptable doses of Cr^{51} is also indirect. The curves of the normal controls, shown in Figs. 2, 3, and 4, can all be adjusted to approximate the true (Ashby) survival rate by the addition of approximately 1+ per cent per day to correct for the radioactivity eluted from the red cells. If the Cr^{51}-labeling had any serious influence on the normal process of blood destruction, it should be indicated by a departure of the apparent survival curves from their exponential dependence on time.

There are distinct advantages to the use of the Cr^{51} method for the study of red-cell survival. The radioisotope method is much more versatile and much more simple than either the Ashby method or the isotopic methods using N^{15}, Fe^{59}, or C^{14}. With the Ashby technique one is restricted to the use of group O donors and group A or B recipients. It is impossible to measure the survival of a patient's cells in his own circulation, and unless the patient under study is group O, it is not possible to observe the behavior of his abnormal cells in a normal individual. Likewise, if the patient belongs to group O, or group AB, it is not possible to study the survival of normal cells in his circulation. From a technical standpoint, also, the Cr^{51} is much simpler to use than the differential agglutination method.

The isotope methods using N^{15}, Fe^{59}, or C^{14} are more flexible than the Ashby, since either genotype-specific, or group O donors can be used. Time is required, however, for the labeling to occur in vivo, so that it is not convenient to employ other donors than those of group O. With these methods it is not possible to measure the survival of the individual's own cells in his own circulation. Technically, the N^{15} method is quite difficult, requiring the use of a mass spectrometer, an expensive and complicated instrument. When Fe^{59} or C^{14} are used the measurements of radioactivity are no more complicated than the detection of Cr^{51}. The iron label, however, can recycle, requiring mathematical treatment of the data.

In contrast, the Cr^{51} method is very simple. The patient's own cells can be labeled in about two hours. In cases with short apparent half-survival

times, the test can be repeated in two or three weeks; and with normal subjects the test could be repeated after fifty to sixty days. Patient's blood can be studied in genotype-specific recipients, and the normal blood of genotype-specific donors can be studied in patients with hemolytic disease. It is possible to measure the half-survival time of a patient's own cells in himself, and then study the survival of normal donor cells. With the well-type counter, the technical details are few, and require only accurate pipetting, accurate measurement of the hematocrit, and reliable radiation detection equipment. We have used both genotype-specific and group O in our studies. It appears from the normal studies, Figs. 2, 3, and 4, that genotype-specific compatible blood has a significantly longer half-survival time than group O blood. This difference is also evident in the Ashby and N^{15} studies where the average survival times are respectively one hundred ten and one hundred thirty days.

SUMMARY AND CONCLUSIONS

1. A method is described for the use of the radioisotope, $NaCr^{51}O_4$, for the study of the rate of destruction or disappearance of red cells from the circulation.

2. The apparent half-survival time has been determined in 14 normal subjects. It ranges from twenty-five to forty days.

3. The Cr^{51} technique can be applied in a variety of clinical situations. It can be used to study the survival of the subject's cells in his own circulation, or elsewhere. It can be used to study the behavior of transfused cells in normal or abnormal subjects.

4. The Cr^{51} label elutes from red cells at a constant rate which approximates 1^+ per cent of the remaining radioactivity per day. Using this as a correction factor, the apparent survival curves obtained with the Cr^{51} method can be adjusted to agree with those obtained by the Ashby or isotopic methods using N^{15}, Fe^{59}, or C^{14}.

5. In the doses used, neither the chromate anion, nor the radioactivity, appears to injure the normal erythrocyte. When the amount of chromate ion used is increased to some 15 times the usual dose, injury to the red cell can be demonstrated. For this reason, it is recommended that the specific activity of the sodium chromate should be at least 100 mc. per gram.

The authors wish to thank Dr. David W. Talmage for advice on many aspects of this work, and Miss Gwendolyn Sirjio for technical assistance.

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RADIOACTIVE SODIUM CHROMATE FOR THE STUDY OF SURVIVAL OF RED BLOOD CELLS

II. The Rate of Hemolysis in Certain Hematologic Disorders

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INCREASED destruction of erythrocytes is an important cause of the anemia which occurs as a complication of many hematologic diseases. Ordinarily, the existence of a hemolytic state can be recognized by the usual clinical and laboratory methods. Such methods provide, at best, a rough approximation of the intensity of the process, and an estimate of the actual or relative rate of hemolysis is a much more difficult problem. A simple and reliable method for the estimation of the rate of hemolysis should be useful for the evaluation of changes that occur spontaneously, or are induced by therapy.

The Ashby technique for the differential agglutination of red cells[1] has been used to study cases of hemolytic anemia, and an increase in the rate of hemolysis of donor cells has been demonstrated. The method is not difficult, but it cannot be applied to the patient's cells in his own circulation. Furthermore, the technique requires the transfusion of group O blood into recipients of groups A or B, which restricts its usefulness. A more versatile, but also a more complicated, method involves the use of isotopically-labeled blood from compatible or universal donors. Hemoglobin precursors labeled with N^{15} or C^{14}, or Fe^{59}, must be administered to a suitable donor several weeks in advance of the experiment. A number of studies[2-5] employing the isotope techniques have been reported, with results that are in agreement with those obtained by differential agglutination. With the Fe^{59} method it is possible to study the turnover rate of iron in patients with hemolytic states, but the technique is cumbersome and requires extended mathematical treatment of the data. In the first paper of this series[6] we demonstrated that radioactive sodium chromate ($NaCr^{51}O_4$) could be used to estimate the apparent survival time of erythrocytes in normal human subjects. The labeling procedure is simple and can be applied to the individual's own cells, or to donor cells, with equal facility. The apparent half-survival time for normal red cells is approximately thirty to forty days. The phrase "apparent survival time" is employed to describe the results obtained with Cr^{51}-labeled cells and to differentiate the results of this method from the results of estimates of the real survival time as determined by differential agglutination or by some of the other isotope methods. The difference between the real and the apparent survival time (or half-survival time) is due to the elution of Cr^{51} from the circulating labeled cells. The elution of Cr^{51} occurs, in vitro, at the rate of approximately 1+ per cent per day and is assumed to

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occur at the same rate, in vivo. When the exponential curve obtained with Cr^{51} is corrected for elution, the result is a good approximation of the linear curve obtained with the Ashby, N^{15}, C^{14}, or Fe^{59} techniques. In the first paper of this series[6] it was suggested that the Cr^{51} method should permit an accurate expression of the rate of hemolysis relative to the normal value. In the present paper we report measurements of the apparent half-survival time of the red cells in several hematologic diseases.

METHODS

The method of labeling the red cells for the clinical studies was described in the first paper of this series, and is a modification of the method of Gray and Sterling.[8] The labeled, washed cells that were injected contained 100 to 150 μc of Cr^{51}, as $NaCr^{51}O_4$.* This amount of radioactivity is substantially less than the maximum permissible human dose of 390 μc recommended by the Subcommittee on Permissible Internal Dose of the National Committee on Radiation Protection.[7] The specific activity of the sodium chromate solution was sufficiently high that the amount of chromium metal injected did not exceed 10 micrograms per milliliter of washed red cells. Donor blood to be studied was collected in ACD solution no longer than twenty-four hours before labeling, and was always genotype-specific, compatible blood. When the subject's own blood was studied, it was either drawn using a heparinized syringe, or allowed to flow freely into heparin or ACD. The cells were labeled immediately after the blood was drawn, and the washed, labeled cells, resuspended in salt solution, were injected slowly into an antecubital vein using an 18-gauge needle and a 100 ml. syringe. Samples for counting were collected at appropriate intervals in heparinized tubes. The hematocrit was determined immediately, and duplicate aliquots of 3 ml. for counting were transferred with siliconed pipettes. The radioactivity of each sample was counted in duplicate either in a Texas-type well counter, or in a well-type scintillation counter. The average net counts per minute per milliliter of the sample, corrected for the hematocrit, is divided by the net counts per minute per milliliter of the standard, and the resulting fraction is taken to represent the fraction of labeled red cells remaining in the circulation. The data are plotted on a logarithmic scale of ordinates against time to demonstrate the apparent half-survival time of the red cells.

Standard hematologic procedures were used for red cell counts and the determination of hemoglobin and hematocrit. The reticulocytes were counted on blood films stained with Brilliant Cresyl Blue. The direct Coombs' test was performed on fresh blood at room temperature, using potent, absorbed antisera. The excretion of fecal urobilinogen was determined on three-day collections of stools, using the semiquantitative method of Watson.[9] The results were expressed in Ehrlich units.

RESULTS

I. *Chronic Lymphatic Leukemia.*—Three cases of chronic lymphatic leukemia, established by lymph node and bone marrow biopsy, were chosen for study with this technique. They represented three different stages of the hemolytic complication of the disease, as determined by the hemogram, reticulocyte count, Coombs' test, and fecal urobilinogen excretion. All were studied using genotype-specific, compatible blood.

CASE 1.—C. M., Unit No. 553002, a 74-year-old white man, developed chronic lymphatic leukemia two years ago. He was asymptomatic, and had never received any blood transfusions. The only treatment he had received was triethylene melamine two years ago, and

*The radioactive sodium chromate used in these studies was obtained from the Abbott Laboratories on allocation from the Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tenn.

3.0 mc. of radiophosphorus four months before the present admission. Except for a slight decrease in the hemoglobin and the red cell count, the laboratory findings were normal.

CASE 2.—W. B., Unit No. 553186, a 52-year-old white man, was admitted for x-ray therapy to his enlarged spleen. He was known to have chronic lymphatic leukemia for two years. He had received x-ray therapy to enlarged lymph nodes eighteen months ago; and was given a course of nitrogen mustard six months ago. During the previous admission, six months ago, he was given two blood transfusions. Physical examination revealed general lymphadenopathy, moderate hepatomegaly, and marked splenomegaly. Laboratory studies showed a moderate normochromic anemia and slight reticulocytosis.

CASE 3.—F. T., Unit No. 564309, a 60-year-old white man, was found to have chronic lymphatic leukemia four and one-half years ago when a routine blood count was made. His first admission to the University Clinics was two months ago, at which time his only symptom was easy fatigue. At that time laboratory studies were: hemoglobin, 13.5 Gm.; red blood cells, 4.2 million per cubic millimeter; reticulocytes, 2.2 per cent; fecal urobilinogen, normal;

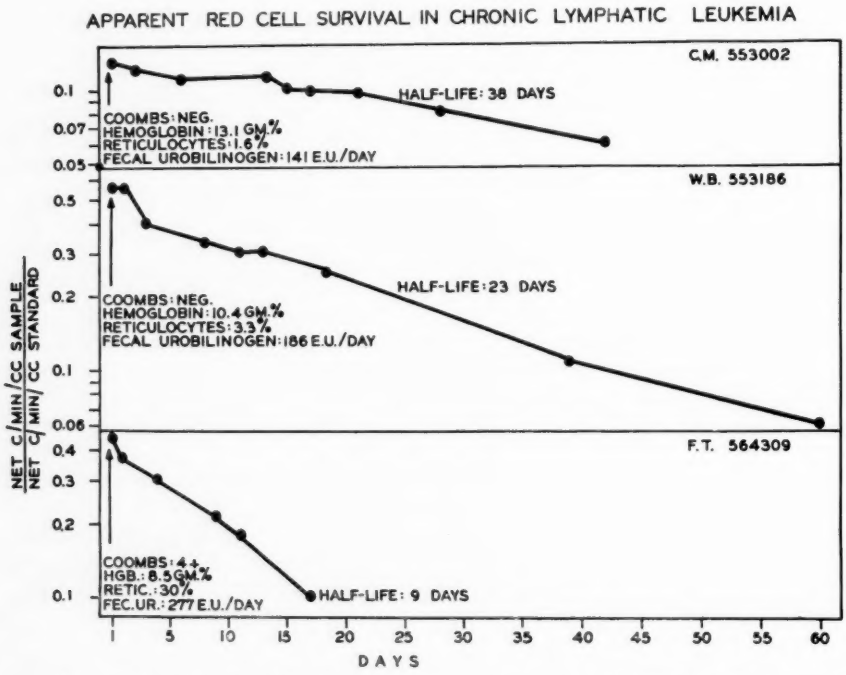

Fig. 1.—Apparent red-cell survival in chronic lymphatic leukemia. *Upper curve:* Case 1; *Middle curve:* Case 2; *Lower curve:* Case 3. In each case genotype-specific compatible blood was labeled and injected shortly after the blood was drawn. The scale of the ordinate is the same for all Figures.

and Coombs' test, negative. The spleen was just barely palpable. He was treated with 3.0 mc. of radiophosphorus. Two months later he was readmitted complaining of increasing weakness and pallor. The spleen was enlarged, there was a marked normochromic anemia, the reticulocytes were 30 per cent, the fecal urobilinogen was definitely elevated, and the direct Coombs' test was 4 plus.

The results of the studies of red-cell survival using Cr^{51} are shown in Fig. 1. It can be seen that the apparent survival time agrees with the clinical impression of the rate of red cell destruction as based on the usual routine laboratory tests. In Case 1, the apparent half-survival time was thirty-eight days, which is a normal result for the Cr^{51} method. Berlin and Lawrence,[3] using red cells labeled

with carbon-14, have also reported a normal survival time in a patient with chronic lymphatic leukemia who was free of symptoms. The same authors reported decreased red-cell survival times in two other patients with chronic leukemia, both of whom were anemic. In our Case 2, the apparent half-survival time for erythrocytes was twenty-three days, which is approximately two-thirds of the normal value. In this patient, on the basis of the routine laboratory studies, it was suspected that the rate of blood destruction was abnormally rapid. It should be possible for a normal bone marrow to compensate for this degree of increased blood destruction, but Case 2 apparently remains anemic because of the inability of his lymphocyte-infiltrated bone marrow to produce additional red cells. In patients of this type, it is evident that the persistent anemia is the result of increased blood destruction and the inability of the bone marrow to increase production because of the leukemic infiltration. It is interesting to note that this patient had splenomegaly out of proportion to the enlargement of the liver and the lymph nodes. In our experience, such patients may respond favorably to therapy with cortisone even though the Coombs test is negative. Case 3 is a typical example of severe hemolytic anemia complicating a chronic leukemia. It should be noted that the patient displayed no evidence of increased red-cell destruction on the previous admission two months earlier. In the interval, the Coombs' test became positive and the hemolytic process was easily recognized by the usual laboratory tests. The apparent half-survival time of erythrocytes in this patient was only nine days, or approximately one-fourth of the normal value. It is proper to express this finding the other way, and to say that red cell destruction is occurring at four times the normal rate. It is not surprising that his bone marrow, infiltrated with leukemic cells, is not able to compensate for this rate of blood destruction. Case 3 is now receiving cortisone, with good effects, and his apparent survival time will be studied again using Cr^{51}.

II. *Myeloid Metaplasia.*—Three cases of myeloid metaplasia, diagnosed on the basis of spleen and liver biopsies, were studied using the Cr^{51} technique. Compatible donor blood was used for each study.

Case 4.—L. B., Unit No. 209806, a 53-year-old white man, was known to have an enlarged liver and spleen for fourteen years, and had had a definite anemia for twelve to thirteen years. In spite of frequent investigations, an etiologic diagnosis had not been made. The diagnosis of myeloid metaplasia was established by biopsy of the spleen in 1951. The anemia has progressed slowly over the years, and at the time of the present study the hemoglobin was 6.6 Gm.; the red blood cells were 2.7 millions per cubic millimeter; the white cells were 17,850, with immature white cells in the peripheral blood; the reticulocytes were 2.2 per cent, and fecal urobilinogen excretion was 185 Ehrlich units per day. The spleen and liver filled the entire abdomen on physical examination.

Case 5.—J. S., Unit No. 557300, a 62-year-old white man, entered the hospital for evaluation of hypertension and enlargement of the liver and spleen. Laboratory studies showed hemoglobin, 16.7 Gm.; red cells, 5.5 millions per cubic millimeter; white cells, 11,100 per cubic millimeter, with immature white cells in the peripheral blood; hematocrit, 49 per cent; reticulocytes, 1.3 per cent; and platelets ranging from 400,000 to over 1,000,000 per cubic millimeter. Aspirated material and sections of bone marrow showed myeloid hyperplasia which was not considered typical of polycythemia vera. Biopsy of the spleen and the liver revealed extensive myeloid metaplasia.

CASE 6.—V. M., Unit No. 565226, a 51-year-old white woman, had complained of fever and weight loss for six months. On physical examination, the principal finding was enlargement of the liver and the spleen. The roentgenogram of the chest was normal. Laboratory studies showed a moderate normochromic anemia, a leukocytosis, marked reticulocytosis, and white count of 7,900 with immature red and white cells in the peripheral blood. Sections obtained by surgical biopsy of the sternal bone marrow revealed myelofibrosis; and the biopsy of the spleen demonstrated myeloid metaplasia, and a nonspecific type of granuloma from which no pathogenic organisms could be cultured. Since the completion of the Cr^{51} study, the patient has been treated with streptomycin which has resulted in a remission of the fever, a feeling of well-being, and a return of the hemogram toward normal.

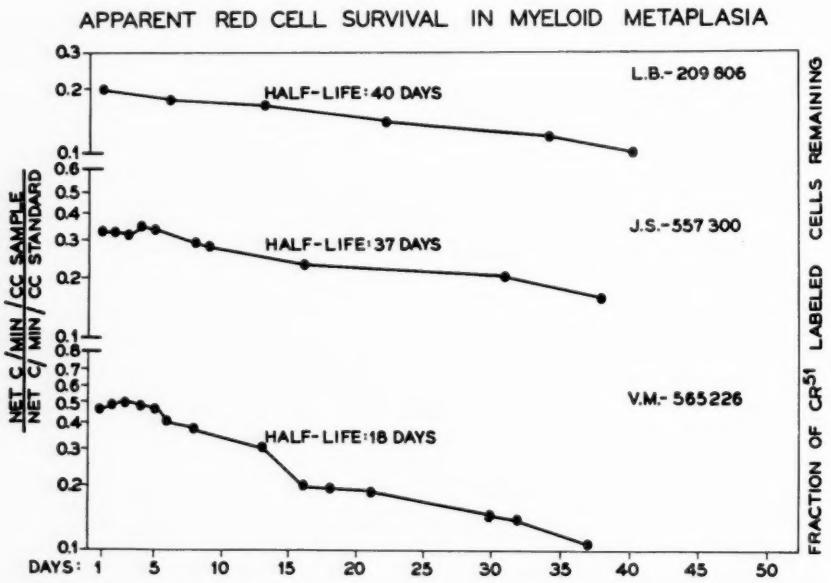
APPARENT RED CELL SURVIVAL IN MYELOID METAPLASIA
L.B.- 209 806
HALF-LIFE: 40 DAYS
J.S.- 557 300
HALF-LIFE: 37 DAYS
V.M.- 565226
HALF-LIFE: 18 DAYS
NET C/MIN/CC SAMPLE / NET C/MIN/CC STANDARD
FRACTION OF CR51 LABELED CELLS REMAINING
DAYS: 1 5 10 15 20 25 30 35 40 45 50

Fig. 2.—Apparent red-cell survival time in patients with myeloid metaplasia. *The upper curve:* Case 4; *Middle curve:* Case 5; *Lower curve:* Case 6. In each case genotype-specific blood was used for the study of red-cell survival.

The results of the studies of red-cell survival using Cr^{51} are shown in Fig. 2. In Cases 4 and 5, the apparent half-survival time was normal, 40 and 37 days, respectively, while in Case 6, the transfused red cells disappeared from the circulation at approximately twice the normal rate. These variations in the rate of red cell destruction are of interest, since each case of myeloid metaplasia appeared to have a different etiological basis. Since the patients' own blood was not studied, nothing can be inferred regarding the survival time of erythrocytes manufactured, at least in part, outside of the bone marrow. To our knowledge, this is the first report of a study of the rate of blood destruction of transfused cells in patients with histologic evidence of extramedullary hematopoiesis. On the basis of these few cases, we can say only that histologic evidence of myeloid metaplasia is not a priori proof that the viability of the erythrocytes of such patients is abnormal. The fate of the patients' own red cells in these cases of myeloid metaplasia is now under investigation, using the Cr^{51} technique.

III. *Acquired Hemolytic Anemia*, not complicating leukemia or lymphoma.

Two cases of acquired hemolytic anemia, not complicating a known neoplastic process, have been studied. One is considered to be an example of idiopathic acquired hemolytic anemia, and the other is secondary to a severe chronic infectious process.

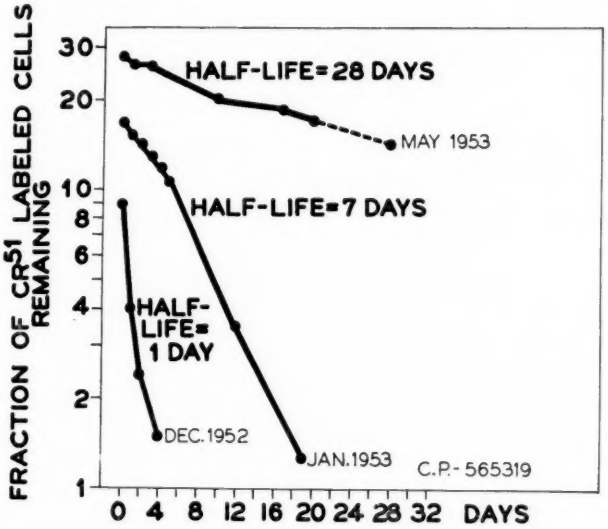

Fig. 3.—Apparent red-cell survival in idiopathic acquired hemolytic anemia: Case 7. The first two studies, December, 1952, and January, 1953, were made with genotype-specific blood from the same donor. The last study was performed with the patient's own blood. Cortisone therapy was started after the first study, December, 1952, and continued through May, 1953. The ordinates are arbitrary values, as is the position of each curve. The time in days refers to the time after Cr^{51}-labeling for each study.

Case 7.—C. P., Unit No. 565139, a 60-year-old woman, was admitted to the University Clinics because of jaundice, weakness, and dyspnea of six months' duration. There was no history of exposure to toxic chemicals or drugs. She had received five blood transfusions shortly before admission. On physical examination the patient was found to be jaundiced, and to have an enlarged liver and spleen. The laboratory data were: hemoglobin, 4.0 Gm.; red cells, 1.2 millions per cubic millimeter; reticulocytes, 43 per cent; serum bilirubin 0.3 mg. for the one-minute and 4.4 mg. per 100 ml. for the total; fecal urobilinogen excretion, 1950 Ehrlich units per 100 gm. of feces; and Coombs' test, 4 plus. Spherocytosis was not observed. Bone marrow biopsy showed marked erythroblastic hyperplasia. Spleen biopsy revealed clumps of normoblasts and erythroblasts, and a tremendous increase in iron deposition. After the hemoglobin was raised to 8.8 Gm. by blood transfusion, the red-cell survival was studied using Cr^{51}-labeled cells from the same donor, before and nine days after the start of effective cortisone therapy. A third study was performed, after the patient had been treated successfully with cortisone for four months. At that time the hemogram was normal, and the patient felt entirely well.

Case 8.—F. H., Unit No. 317985,* a 59-year-old woman, has been admitted to the Clinics many times during the past eighteen years, because of recurrent regional ileitis. On a number of occasions resection of diseased segments of bowel has been necessary. In addition, she has a chronic renal disease, probably glomerulonephritis, which is responsible

*We are indebted to Prof. Walter L. Palmer for permission to include this patient in our study, and in this report.

for a slowly increasing azotemia. In 1949 she was first found to have a progressive anemia, accompanied by recurring bouts of jaundice, and poor response to blood transfusions and other forms of therapy. Her clinical course has consisted of frequent exacerbations and remissions of the gastrointestinal and hematologic diseases. At the time of the present study, she was hospitalized because of anemia, fatigue, and dyspnea. The relevant laboratory data were: hemoglobin, 8.0 Gm.; red cells, 2.5 millions per cubic millimeter; reticulocytes, 2.2 per cent; serum bilirubin, 0.3 mg. for the one-minute value, and 1.8 mg. for the total; fecal urobilinogen excretion, 170 Ehrlich units per day; and a normal result for the Congo Red test. The stools were negative for blood by the benzidine reaction throughout the period of the study. After the Cr^{51} study, the patient was placed on cortisone therapy with some improvement in the severity of the anemia.

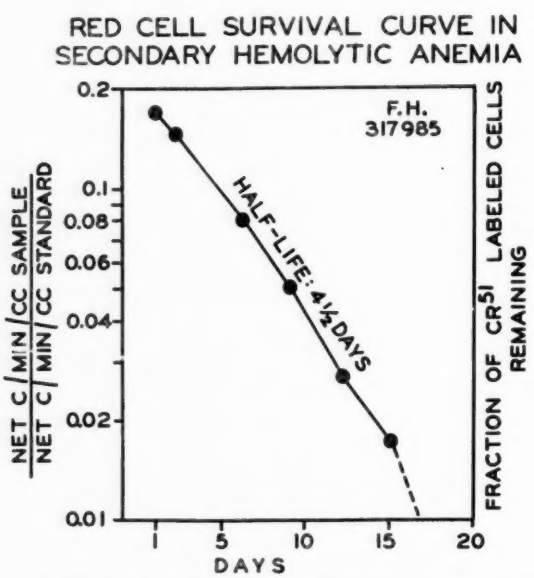

Fig. 4.—Apparent red-cell survival in secondary hemolytic anemia: Case 8. This study was performed using genotype-specific compatible blood.

The markedly increased rate of blood destruction in these patients is shown in Figs. 3 and 4. In Case 7 (Fig. 3) the apparent half-survival time was only one day, before the administration of cortisone. After nine days of therapy another study was initiated using labeled blood from the same donor, and at this time the apparent half-survival time was seven days, which is to say that the rate of blood destruction was only one-seventh as rapid as before treatment. The seven-day apparent half-survival time is still much faster than the normal, by a factor of approximately 5. At the conclusion of the second Cr^{51} study, the hemogram was approaching normal, and three months later when a third measurement of red-cell survival was done, the blood counts were normal. At this time, the Coombs test was 1 plus, and there was still an increase in reticulocytes. The apparent half-survival time had increased to twenty-eight days, which is a normal value. It is proper to say that cortisone therapy in this case has decreased the rate of blood destruction by a factor of 28. It is also possible to say that therapy apparently has corrected the defect responsible for the abnormal hemolysis.

In Case 8, the apparent half-survival time was four and one-half days, or about one-eighth of the normal value. This is equivalent to saying that this patient destroyed red cells at eight times the normal rate. The Cr^{51} technique was of particular interest in this case, because it demonstrated that abnormal blood destruction was a factor in the persistence of the anemia. Ordinarily, the

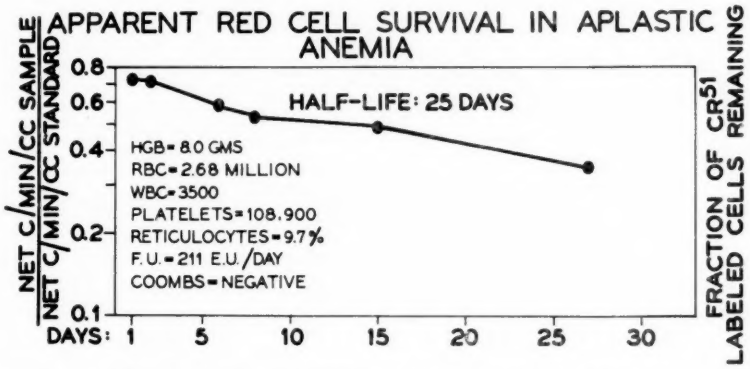

Fig. 5.—Apparent red-cell survival in primary refractory anemia: Case 9. This study was performed using genotype-specific compatible blood.

anemia of such a patient would be explained as due to the combination of blood loss, infection, and azotemia. It was a surprise to find the evidence for a hemolytic component in the anemia of this patient, and this finding justified a more diligent trial of cortisone therapy than would have been undertaken otherwise.

IV. *Aplastic Anemia.*—One case of primary refractory anemia with histologic evidence of an aplastic bone marrow has been studied.

Case 9.—O. W., Unit No. 440705, a 27-year-old man, was known to have refractory anemia for at least one and one-half years. There was no history of exposure to toxic chemicals. Over this period his hemoglobin ranged from 7.0 to 9.0 Gm., and he required blood transfusions at intervals of five to eight months. Physical examination was normal except for the pallor. The laboratory studies showed: hemoglobin, 8.0 Gm.; red cells, 2.7 million per cubic millimeter; white cells 4,500 per cubic millimeter; platelets, 110,000 per cubic millimeter; reticulocytes, 9.7 per cent; osmotic fragility, normal; Coombs' test, negative; fecal urobilinogen excretion, 211 Ehrlich units per day. Surgical biopsy of the bone marrow was done, and the sections displayed severe hypoplasia of the marrow elements without fibrosis. The patient's own blood was labeled with Cr^{51} and reinjected to study erythrocyte survival.

The results of the study are shown in Fig. 5. The apparent half-survival time for this patient's own blood was twenty-five days, which is about 70 per cent of the normal value. This means that the patient's cells were being destroyed somewhat more rapidly than in the case of normals, a situation which is obviously a disadvantage in association with a hypoplastic bone marrow. The only other report on the survival time of red cells in aplastic anemia is the study of Baar,[10] who calculated the erythrocyte-survival time from the rate of stercobilin excretion in one case. He found a relative increase as compared with the normal. It will be of interest to perform more studies on other patients with aplastic anemia.

DISCUSSION

The radiochromium technique for studying the apparent survival time of red cells in vivo has been employed in nine patients with a variety of blood diseases. The results presented here demonstrate that the method is entirely satisfactory for the study of the severity of the hemolytic process. The necessary measurements are simple to perform, and the Cr^{51}-labeling can be done easily and safely, using either donor blood or the patient's cells. Although an absolute value for the rate of red-cell destruction cannot be obtained by this method, except by extrapolation, the relative rate of hemolysis with respect to normal subjects, or before and after therapy in the same subject, can be determined readily and reliably. The importance of the method depends upon its simplicity as a means of obtaining quantitative information about hemolytic states. Other workers have used the differential agglutination techniques, or other isotope methods, to study patients with hemolytic processes, and comparable data have been obtained. However, none of the other methods are as well suited to routine clinical use as the Cr^{51} technique.

CONCLUSIONS

1. Red cells labeled with Cr^{51} have been used to measure the apparent half-survival time of erythrocytes in patients with lymphatic leukemia, myeloid metaplasia, acquired hemolytic anemia, and primary aplastic anemia.

2. The Cr^{51} method provided a quantitative expression of the rate of blood destruction in frank hemolytic states which was in agreement with clinical judgment based on the usual laboratory procedures.

3. In one case of lymphatic leukemia, one case of myeloid metaplasia, and in a patient with chronic regional ileitis the Cr^{51} method demonstrated the existence of a state of abnormal blood destruction which had not been suspected.

The authors wish to thank Dr. David W. Talmage and Miss Gwendolyn Sirjio for valuable technical assistance in the prosecution of these studies.

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BLOOD ISOTOPE LEVELS FOLLOWING A TEST MEAL OF I^{131}-LABELED PROTEIN

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PREVIOUS studies[1, 2] have shown that impaired protein digestion in persons with marked pancreatic insufficiency may be determined by measuring the fecal excretion of isotope following a test meal of I^{131}-labeled protein. Although blood levels of isotope in these patients were generally below those in persons with normal digestion, the rate of absorption in both groups was so slow that all values were too low to make the difference between them significant. In a number of additional observations, utilizing varying quantities of nonradioactive protein in the test meal, the rate of absorption of ingested isotope by normal persons was found to be roughly inversely proportional to the amount of administered protein.

This communication is concerned with observations made with an amount of nonradioactive protein in the test meal which resulted in maximum isotope blood levels in persons with normal digestion, two to three hours after its administration. Under these conditions blood isotope curves were found to be potentially sensitive to defects in the digestive process.

METHODS

The test meal was prepared essentially in the same manner described previously,[2] except that commercially available I^{131}-labeled albumin* was substituted for labeled casein, and gelatin was substituted for nonradioactive casein. Each patient received 0.5 Gm. of gelatin per kilogram of body weight and 100 μc of iodinated albumin, mixed in 200 ml. of distilled water.

Sixteen persons with normal protein digestion were used as controls. Nine of these presented no abnormality of the digestive system, and seven were persons who had previously undergone subtotal gastrectomy for peptic ulcer. The latter had been studied previously on the Metabolic Ward in the University Hospitals for protein and fat absorption,[3] and were found to have no abnormality in that regard. Three individuals with marked pancreatic insufficiency were used as contrasting clinical material. In all three, pancreatic insufficiency was severe enough to necessitate pancreatic extract for maintenance of weight and control of diarrhea. Each had been previously studied[2] with I^{131}-labeled casein, and by this technique were found to have excessive loss of isotope in the stools.

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*The I^{131}-labeled serum albumin was obtained from Abbott Laboratories, Chicago, Ill., upon authorization of the AEC.

Lugol's solution (45 minims daily) was given to each patient for three days prior to the test meal, and during this period pancreatic extract was not taken by the three with pancreatic insufficiency. After overnight fast, each patient drank freshly prepared test meal through a glass straw, and oxalated blood samples were drawn at one, two, and three hours after the test meal. The radioactivity in the blood samples was determined with a scintillation counter, and the isotope content of the total blood volume (assumed to be 7.7 per cent of body weight) was calculated and expressed as percentage of ingested isotope.

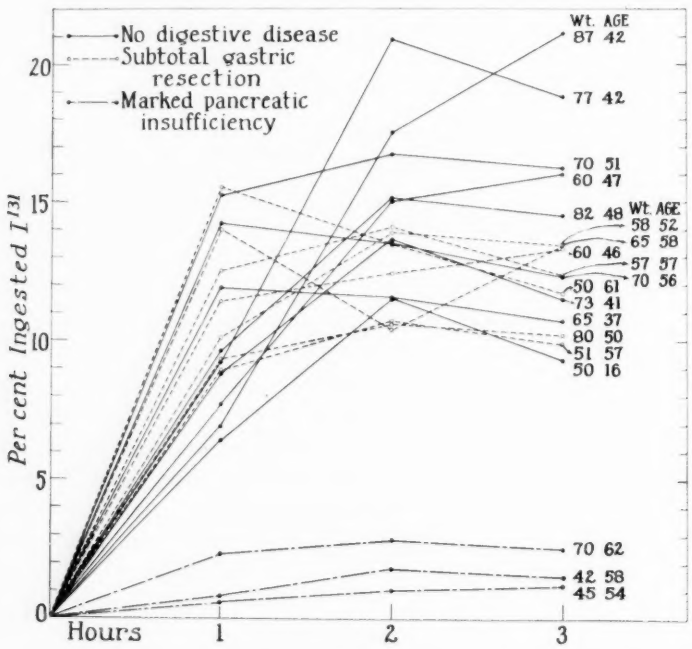

Fig. 1.—Blood isotope levels for three hours after administering a test meal of I^{131}-labeled protein to nine persons without digestive disease, seven persons with subtotal gastric resection, and three persons with marked pancreatic insufficiency.

RESULTS

In persons with normal digestive processes, the isotope content of the blood rose sharply during the first one to two hours following the test meal and then leveled off (Fig. 1). With only one exception maximum blood levels were obtained within three hours after the test meal, and at two hours following the test meal none of these patients had blood values less than 10 per cent of ingested isotope. In contrast to these results, three patients with pancreatic insufficiency showed a much lower isotope content of the blood, none exceeding 3 per cent of the ingested dose. Delayed gastric emptying as the cause of these low values would seem to be eliminated by the fact that maximum blood values were obtained in the same time sequence as in the normals.

DISCUSSION

Multiple mechanisms are involved in the digestive process. As components of it, digestion and absorption are two different functions, and the rate with

which each proceeds does not necessarily parallel its extent. A simplified means whereby each of these various functions might be evaluated accurately should aid in a better understanding of the digestive process.

Results of this study suggest that the technique herein described may be useful in the evaluation of protein digestion and absorption. Since by this method a normal rate of isotope entrance into the blood indicates that the rate of both digestion and absorption is normal, low blood isotope values could result from a defect in the rate of either digestion or absorption, or both. As a supplementary procedure, protein absorptive processes per se might be evaluated by measurement of the rate and extent of entry into the blood of ingested I^{131}-labeled amino acids. As test substances, they would seem to have an advantage over nonradioactive amino acids[4] in that the metabolic effects of the liver and other tissues on blood amino acid levels would be minimized.

Previous studies[1, 2] have shown that the extent of protein digestion may be determined by the fecal excretion of isotope following a test meal of I^{131}-labeled protein. By utilization of a test meal of lower protein content described in this communication, it would seem likely that defects in both the rate and extent of protein digestion or absorption might be detected in one simplified procedure.

SUMMARY

Blood isotope levels for three hours following a test meal of 100 μc iodinated albumin and 0.5 Gm. gelatin per kilogram of body weight serve to distinguish the rate of protein digestion and absorption in 16 persons with normal digestive processes from that in three persons with marked pancreatic insufficiency.

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SEVERE CARBON TETRACHLORIDE POISONING WITH RECOVERY FOLLOWED BY FATAL HOMOLOGOUS SERUM HEPATITIS

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CARBON tetrachloride poisoning continues to be a significant clinical and public health problem. The increasing industrial importance of this substance as an organic solvent is based on its complete non-flammability and other highly useful physical and chemical properties. In spite of the widespread industrial use of carbon tetrachloride, it is stated that serious poisoning due to this solvent is less common in industry than among incidental and household users.[1, 2] On the whole it appears that in industry, protective controls are instituted and enforced; preplacement medical examinations are used to screen away highly susceptible individuals. The incidental and household users, on the other hand, have no warning of the serious toxic effects of this substance, and indeed in some instances of solvent mixtures and brand-name household cleaning fluids, the individual user may be unaware of the presence of carbon tetrachloride in the cleaning fluid itself.

The case presented below is that of a severe carbon tetrachloride poisoning with hepatitis and uremia, in whom the poisoning occurred by accidental ingestion. This patient recovered from the poisoning with the assistance of parenteral nourishment, the careful administration of fluids and alkali, and three exchange whole blood transfusions given at the most critical stage of uremia. Three and one-half months later, however, this patient developed a fulminant and fatal homologous serum hepatitis.

CASE PRESENTATION

C. P., Hospital No. 87482, was a 32-year-old man admitted to the Medical Service on June 30, 1951. Two days before admission, while cleaning furniture on his job, he accidentally drank approximately two ounces of carbon tetrachloride, mistaking this colorless liquid for water which he had in a second container. This was followed almost immediately by recurrent vomiting. Shortly thereafter he developed headache, severe stabbing ocular pains and urinary frequency and urgency with hematuria. Three or four hours later his vomitus contained large quantities of blood-tinged fluid.

The nausea and vomiting continued intermittently during the succeeding two days prior to hospitalization. Later it was learned that about two hours prior to the ingestion of the carbon tetrachloride the patient had consumed at least one-half pint of whiskey, and that for the past ten years he had regularly consumed excessive quantities of alcohol. The history, however, did not suggest previous clinical liver disease.

On admission the patient was a well-developed muscular man, cooperative, and not in acute distress. He complained of abdominal and lumbar pain. His temperature was 103° F.; blood pressure 140/90; pulse 88. He was dehydrated. The skin and mucous

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membranes had an icteric color. The liver was tender and enlarged to palpation about 5 cm. below the costal margin. The heart, lungs, and remainder of the physical examination were negative.

The laboratory data on admission showed: blood nonprotein nitrogen, 124 mg. per cent, icteric index 27 units, serum chloride 106 milliequivalents per liter, prothrombin time 58 per cent, hematocrit 46 per cent.

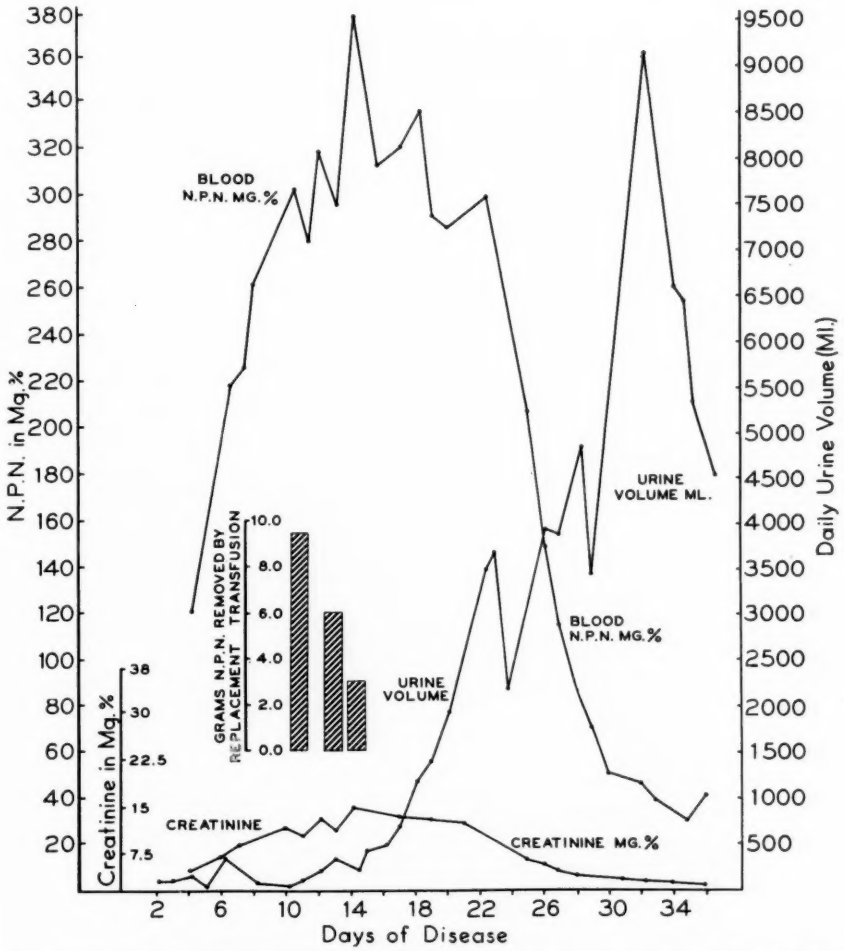

Fig. 1.—Blood chemical changes, urine volume, and the effect of exchange transfusions in carbon tetrachloride poisoning.

During the first week of hospitalization jaundice became severe, serum bilirubin 150 mg. per cent, and the liver became progressively larger. During the second week, however, there was a rapid regression both in the serum bilirubin level and in the hepatomegaly. At the end of the third week clinical jaundice was no longer present. The kidneys, on the other hand, presented a much more serious picture. From the third through the fourteenth day of illness, the urinary output averaged less than 400 Ml. per twenty-four hours, and on several days less than 100 ml. daily. The urine showed a low fixed specific gravity and many red and white blood cells.

On the seventh day of illness, the syndrome of uremia was fully developed, including vomiting, diarrhea, and uremic frost. The blood nonprotein nitrogen and creatinine increased steadily, reaching a peak of 384 mg. per cent and 38 mg. per cent, respectively, on the thirteenth day. Acidosis was marked, the CO_2 combining power falling to 23 volumes per cent. There was a progressive rise in blood pressure from 140/90 on admission to 210/120 on the twelfth day of illness.

On the eleventh day of illness, the patient's state was critical; he showed clinical uremia and hyperpotassemia. The serum potassium and sodium were 7.5 and 84 milliequivalents per liter, respectively. At this stage the first of three replacement transfusions was done. A total of 4.0 liters of blood were withdrawn, resulting in the removal of 9.6 Gm. of nonprotein nitrogen. The patient showed immediate clinical improvement.

On the thirteenth day of illness, the patient's condition was again critical. An exchange transfusion at this time resulted in the removal of 6.2 Gm. of nonprotein nitrogen. Shortly after the completion of this transfusion, the patient developed pulmonary edema but improved gradually after intravenous digitalization. On the following day the serum potassium was 10 milliequivalents per liter. The third replacement transfusion was done at this time without the development of pulmonary edema. Twenty-four hours later, the fifteenth day of illness, the patient began a spontaneous diuresis reaching a peak of 6.8 liters several days later. Symptomatic improvement paralleled the diuresis. There was a gradual fall in blood pressure to 124/84. The blood chemical findings, urinary output, and the results of exchange transfusions are summarized in Fig. 1.

On the forty-sixth day of illness the patient was discharged from the hospital with no evidence of renal or hepatic insufficiency.

During the succeeding twelve weeks the patient remained asymptomatic. Fourteen weeks after the last transfusion, the patient was readmitted because of arthralgia, fever, nausea and vomiting, and jaundice. He had not had contact with alcohol or carbon tetrachloride since discharge from hospital. On admission, the temperature was 105° F., heart rate 100, and respiration 20. The urine was positive for bile, negative for protein and sugar, and had specific gravity of 1.025. The patient was well nourished but deeply jaundiced and was exquisitely tender over the liver. The clinical impression was homologous serum hepatitis, severe with probable acute yellow atrophy of the liver. The significant blood chemistry findings during the first and second hospital admission other than nonprotein nitrogen are summarized in Table I. It is to be noted that the blood chemical data returned essentially to normal values following carbon tetrachloride poisoning. During the second admission after development of serum hepatitis severe depression of prothrombin and cholesterol esters was observed. He was treated with parenteral glucose, vitamin B_1 complex, and choline. His temperature continued between 103° and 105° F. for the first three days and he became mentally confused and somnolent. At this time ACTH, 60 mg. every six hours, and intravenous terramycin were administered, following which his temperature fell to normal. He developed marked generalized abdominal

TABLE I. BLOOD CHEMICAL DATA AS RELATED TO LIVER INVOLVEMENT*

DATE	ICTERIC INDEX	SERUM BILIRUBIN		SERUM CHOLESTEROL		PROTHROMBIN (%)	PLASMA ALBUMIN (GM. %)
		DIRECT	TOTAL	TOTAL	ESTERS (%)		
7/2	27					58	
7/5		100	150				2.95
7/9						81	2.56
7/12	56			86			1.69
7/16		9.6	11.4				3.21
8/6	13	0.5	0.7			85	3.70
10/22	118	22	40	136	23		
10/25	183	37	58	118	9	16	

*First admission July 2, 1951 through Aug. 6, 1951. Second admission, following serum hepatitis Oct. 22, 1951.

tenderness but was judged not to have an acute surgical abdomen by the surgical service. He continued progressively downhill and died on the sixth hospital day.

AUTOPSY REPORT

The autopsy was performed out of our jurisdiction two days after death; however, opportunity was offered one of us to observe as well as to obtain blocks of some of the tissues.

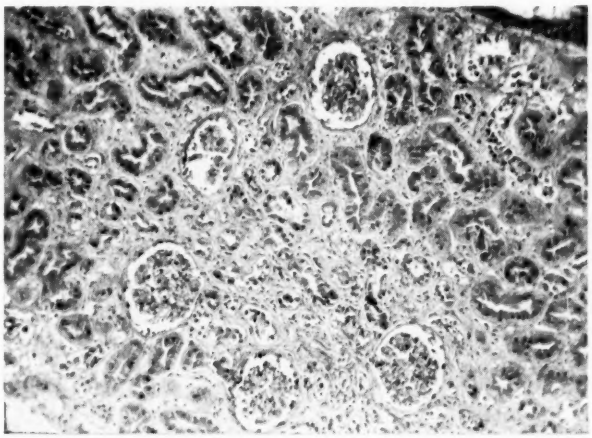

Fig. 2.—Kidney showing diffuse fibrosis of the cortex and slight thickening of Bowman's capsule of some glomeruli. The tubular changes present were interpreted as both antemortem and postmortem in origin.

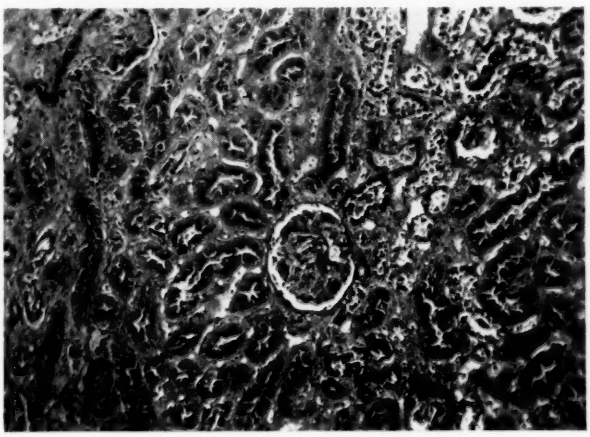

Fig. 3.—Kidney, showing in addition basophilic granular deposits replacing the epithelium of the convoluted tubules.

The body was deeply stained by bile, and when the abdomen was opened considerable digestion of peripancreatic fat was seen. The liver was pale and markedly reduced in size.

Sections from the kidney showed generalized cortical fibrosis with areas of scarring. Some of the glomeruli showed thickening of Bowman's capsule, and a few were hyalinized. The tubules showed lysis, and in some areas a basophilic granular cytoplasmic deposit was found (Figs. 2 and 3).

When examined, the pancreatic sections disclosed hemorrhage of the peripancreatic tissue as well as saponification of the fat. This extended into the interstitial substance. The parenchymatous tissue revealed extensive lysis and in some areas an infiltration of inflammatory cells (Fig. 4). The most striking changes were seen in the sections of the liver. Here widespread necrosis of the liver cord cells was present, most marked about the central veins, associated with disorganization of the normal liver architecture. The remaining cord cells showed considerable variation in the size of the nuclei and in some instances multinucleation. Lymphocytic infiltration was present in the portal areas and there was proliferation of bile ducts (Fig. 5).

The following diagnoses were established: Massive necrosis of the liver; hemorrhagic necrosis of the pancreas (pancreatic necrosis is known to occur in some cases of severe acute hepatitis[3]); lobular pneumonia with hemorrhage; chronic passive congestion and edema of the lungs.

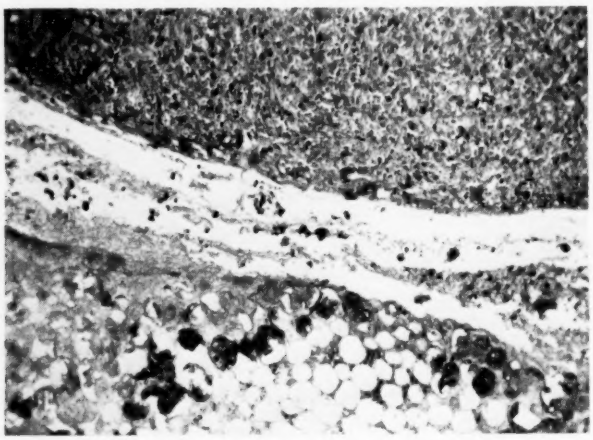

Fig. 4.—Pancreas; autolysis obscures the changes in the parenchyma. The adjacent tissues show edema, hemorrhage, and fat necrosis.

DISCUSSION

The death of this patient from serum hepatitis was especially tragic, since careful therapy had saved his life from what appeared to be an almost certain carbon tetrachloride fatality. On the eleventh, thirteenth, and fourteenth days of illness, when the patient appeared moribund, the replacement transfusions were given, with immediate clinical improvement. The volume of blood and fluid administered on the day of the second transfusion exceeded the volume of fluid lost by excretion and venesection, and undoubtedly was responsible for the onset of pulmonary edema. Excessive fluid administration with serious or fatal pulmonary edema and heart failure in the treatment of lower nephron nephrosis have been emphasized by several authors.[4-7]

In the acute toxic nephroses such as carbon tetrachloride nephrosis it is known that tubular regeneration will occur in about twelve days. The therapeutic problem is to keep the patient alive during this interval. The prognosis in such cases has greatly improved in recent years as a result of a better understanding of the importance of fluid and salt restriction in the prevention of pulmonary edema and heart failure; the significance of potassium intoxication

and the availability of several effective methods of extrarenal excretion during the critical two- or three-day period prior to the onset of spontaneous diuresis.

The opinion has been expressed that uremia and electrolyte abnormalities in severe carbon tetrachloride nephrosis are rarely of such magnitude as to cause death.[4] We believe that in some of the cases previously reported, as well as in the case reported here, uremia and hyperkalemia were an immediate threat to the patient's life, necessitating some form of extrarenal excretion. The types of extrarenal excretion which have been most widely used in the management of acute temporary renal insufficiency are peritoneal lavage,[8] intestinal lavage,[9] exsanguination or replacement transfusions,[10-13] and the use of an artificial kidney.[13-16]

Fig. 5.—Liver, showing marked destruction of cord cells. Note the wrinkling of the capsule, increased number of bile ducts, disorganization of cord cells at the periphery of the lobules, and their absence at the center.

The hazards of infection, channelling, hemorrhage, and excessive absorption of fluids constitute serious limitations of the technique of peritoneal lavage.[8] Intestinal lavage[9] has been used as a method of extrarenal excretion, especially in the management of the hyperkalemia of acute temporary renal insufficiency. Excessive fluid absorption is almost inevitable by this method, and may precipitate pulmonary edema. Exsanguination transfusion appears to be a highly effective procedure which requires little expensive equipment and a minimum of personnel. This procedure seems to have been more widely used in Europe than in America.[12] Considerable quantities of nitrogenous waste products, electrolytes (especially potassium) as well as the nondialyzable toxic substances of uremia can be removed by this procedure which may be lifesaving. Especial care must be taken in regard to possible transfusion reactions and overloading of the cardiovascular system with the production of heart failure and pulmonary edema. The most serious hazard which we encountered was the development of transfusion hepatitis. This complication is at present unpredictable and cannot be prevented by ultraviolet irradiation of blood prior to transfusion.[17] Storage of liquid plasma at room temperature for several months is said to inactivate the virus of serum hepatitis, but this procedure cannot be applied to whole blood.[18]

Fortunately, the incidence of hepatitis following whole blood transfusion at present is less than 1 per cent.[19]

The possibility of serum hepatitis following exchange transfusions has been recognized. In one series of 95 cases an incidence of 3 per cent is reported.[20] We have not found a report of any fatal cases following exchange transfusions.

The artificial kidney will undoubtedly be the method of choice in the management of acute temporary renal insufficiency. Recent mechanical improvements in several types of instruments have decreased the hazards of clotting, hemolysis, and pyrogen reactions. The control of blood flow through the apparatus has been improved sufficiently so that this procedure can be used safely in patients with congestive heart failure.[14, 15] Overhydration may actually be corrected when the bath fluid is made hypertonic containing 2.5 per cent glucose.[16] The use of an artificial kidney instead of replacement transfusion in our patient would have prevented the development of the fatal serum hepatitis.

During the three-year period 1949, 1950, and 1951, two hundred seventeen cases of carbon tetrachloride poisoning were reported by the Industrial Commissions of the following states: California, Illinois, Ohio, New York, and Wisconsin.[2] Twenty-three of these cases resulted in fatalities. This number of cases represents compensable injuries and therefore reflects only a fraction of the total cases of intoxication due to carbon tetrachloride, since they do not include incidental and household users. It has been emphasized that fatal poisoning from this solvent occurs most commonly from incidental and household users as contrasted with industry.[1, 2]

In view of the large number of fatal poisonings among nonindustrial users of carbon tetrachloride, the question naturally arises: Are the warning labels on small quantity containers of this solvent adequate for the protection of the public health? The 1935 agreement between the Public Health Service and manufacturers of carbon tetrachloride calls for the following type label:

> "Warning! Volatile Solvent. Vapor Harmful. Use with Adequate Ventilation. Avoid prolonged or repeated contact with skin. Do *NOT* take internally."

This requirement holds, however, only for containers where the quantity of carbon tetrachloride or similar mixed solvents exceeds 450 milliliters. Carbon tetrachloride is so toxic that ingestion of more than 3 or 4 ml. may be fatal.[22] Habitual users of alcohol, obese individuals, and patients with liver or kidney diseases are known to be especially susceptible to minute quantities of carbon tetrachloride. Serious and even fatal poisoning has been reported from simple exposure to the fumes of this solvent inhaled while assisting or closely observing home dry cleaning in a poorly ventilated basement. In most such instances the individual had recently ingested some type of alcoholic beverage. We are in agreement with the opinion expressed by others[24] that carbon tetrachloride packaged in any quantity, large or small, should be labeled with a prominent display of the word *POISONOUS* along with the usual skull and crossbones insignia.

SUMMARY

A case of accidental severe carbon tetrachloride poisoning is described in which recovery appears to have been made possible by means of exchange blood transfusions at a critical stage of uremia. After complete recovery the patient died about four months later from a fulminant homologous serum hepatitis. Limited postmortem examination showed hemorrhagic necrosis of the pancreas and massive necrosis of the liver.

A brief review is presented of the use of exchange transfusions and extrarenal excretion methods (peritoneal and intestinal lavage and the artificial kidney) in the management of lower nephron nephrosis. Homologous serum hepatitis may represent a serious complication of exchange blood transfusions.

Data are presented which indicate that carbon tetrachloride poisoning in industry and among incidental and household users continues to be a significant clinical and public health problem. In five states two hundred seventeen cases of carbon tetrachloride poisoning were recorded for the three-year period 1949 to 1951 with a 10 per cent mortality.

The authors are of the opinion that the public should be more fully informed of the potential serious toxic effects of carbon tetrachloride. Small as well as large quantities of this solvent should be labeled with the prominent display of the word *POISONOUS* in addition to the usual skull and crossbones insignia.

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INFLUENCE OF ADRENOCORTICAL STIMULATION ON ANTIBODY PRODUCTION

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STIMULATION of the suprarenal cortex by adrenocorticotropic hormone (ACTH) is applied therapeutically in many diseases. Many clinicians have found most temporary successes, often even striking, diminished resistance to infections.

The development of unperceived intercurrent infections has been described in the course of ACTH therapy.[2, 16] In other cases an unfavorable influence has been noted on infectious processes,[11] especially on pulmonary tuberculosis.[6, 7] The same effects have been found during ACTH treatment of animals.[17-19]

The cause of the lessened resistance is not yet clear, but it has been ascribed to a decreased activity of leukocytes as a result of depression of the inflammatory reaction. Further, a depression of antibody formation should be considered. It is doubtful whether inhibition of the hyaluronidase also plays a part. The influence of ACTH, or more generally of an increased adrenocortical activity on antibody formation, has been investigated further experimentally. Some authors, notably Doughterty and White, have found an increased antibody formation during ACTH treatment in animal experiments.[3, 4, 9] Others have seen no effects whatsoever in antibody titers.[5, 21] Recently a pronounced decrease of antibody titers, together with a marked atrophy of lymphoid tissue, has been described during ACTH treatment.[1, 8] The latter effect has been generally observed.[20] Observations in the human being do not suggest a decrease,[10, 14] but rather a slight increase[15] of antibody titers following ACTH.

In two series of tests we have endeavored to investigate further the influence of adrenal cortex stimulation on the production of immune substances. In the first series the serum titer was ascertained in previously immunized animals after a single ACTH injection.*

In the second splenic tissue of previously immunized animals red and white pulp was cultured separately, twenty-four hours after an ACTH injection, and the immune body formation of this tissue in vitro compared with that of control animals. These latter received normal saline solution instead of ACTH.

MATERIALS AND METHODS

Twenty young adult chinchilla rabbits were immunized with paratyphoid B-vaccine, rich in H-antigen. They received three weekly injections of, respectively, 0.3 ml., 0.3 ml., and 0.5 ml. of vaccine intravenously. These animals were used in three series of experiments—A, B, and C.

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*The ACTH preparation was kindly supplied to us by N. V. Organon, Oss, Holland.

Experiment A.—Four of these animals received one single injection of ACTH (10 mg.) intramuscularly nine days after the last antigen administration. H-agglutinin titers were determined 0, 3, 8, and 24 hours after the ACTH injection.

Experiment B.—Two other animals underwent the same procedure six months after the last antigen injection.

The readings of the four agglutination tests of each animal were done simultaneously, so as to make them comparable.

Experiment C.—The remaining 14 animals were used for the tissue culture experiments twenty days after the last antigen injection. At this time the maximum antibody production is past and is therefore on the decline again, although the serum titer is still high.

Two rabbits were used for each experiment, one received 10 mg. of ACTH, and the other (control) at the same time received intramuscularly normal saline solution. Both animals were from the same litter, were of similar development, and were treated entirely parallel as regard to previous treatment, temperature, feeding, and as regard to the experiment proper.

To establish a good ACTH response respectively the absence of any adrenocortical stimulation in the control animal's total white blood cell counts and differential counts were made immediately before and 3, 8, and 24 hours after the injection of ACTH or 0.9 per cent NaCl, respectively.

Twenty-four hours after the respective injection both animals were killed by bleeding after having received 40 mg. of heparin intravenously. The spleen was removed aseptically, and the red and white pulp was prepared under a dissecting microscope. Half of the material from each was extracted for antibody content, and the other halves were cultivated in roller tubes in rabbit serum-Tyrode solution (1:4). Agglutination titers of the extract and culture fluids were determined and compared as described earlier[12] to express antibody production in the explants. Control sections of the spleens were made after Zenker-formol fixation. Staining was with methyl green-pyronin according to Unna-Pappenheim (Koch's modification).[13]

RESULTS

Experiments A and B.—Table I shows the course of the serum-agglutinin titers during the twenty-four hours after the injection of 10 mg. of ACTH. These titers do not appear to show any rise such as described by Dougherty and White, but there exists, rather, a tendency to fall.

TABLE I

	BEFORE ACTH	3 HOURS AFTER ACTH	8 HOURS	24 HOURS	TIME AFTER THE LAST ANTIGEN INJECTION
1.	1: 28000	1: 28000	1: 28000	1: 28000	9 days
2.	1: 50000	1: 37500	1: 28000	1: 28000	9 days
3.	1: 15830	1: 15830	1: 15830	1: 15830	9 days
4.	1: 28000	1: 21500	1: 21500	1: 21500	9 days
5.	1: 1280	1: 1280	1: 960	1: 640	6 months
6.	1: 1280	1: 960	1: 960	1: 640	6 months

Experiment C.—A characteristic fall in lymphocyte count and the rise in neutrophil count was observed in all ACTH animals—proof of a good reactivity of the adrenocortex toward the ACTH preparation used. No one of the control animals showed any reaction in the white blood count.

Tables II and III record the results of the tissue culture tests. In all cases the red as well as the white pulp of the ACTH animals has produced more antibodies than that of the corresponding control animals.

TABLE II. RED PULP

	10 MG. ACTH			1 C.C. NACL 0.9 PER CENT		
EXPERIMENT	TITER BEFORE	TITER AFTER	PRODUCTION	TITER BEFORE	TITER AFTER	PRODUCTION
1.	1:16	1:96	5×	1:12	1:48	3×
2.	1:16	1:128	7×	1:16	1:128	7×
3.	1:16	1:192	11×	1:4	1:32	7×
4.	1:8	1:128	15×	1:32	1:128	3×
5.	1:4	1:32	7×	1:8	1:32	3×
6.	1:16	1:256	15×	1:8	1:48	5×
7.	1:16	1:128	7×	1:8	1:32	3×
	Average		9.7×			4.42×

In Table II the results from the red pulp are summarized. The animals of the same experiment appear next to each other, the one with ACTH, the other with NaCl.

The difference in antibody production can be seen very clearly.

It is apparent from the table that in all the experiments the ACTH animals produce more antibodies than the control animals. The average production of the ACTH animals is 9.7×, that of the control animals 4.42×.

The values for the white pulp are given in the same manner in Table III.

TABLE III. WHITE PULP

	10 MG. ACTH			1 C.C. NACL 0.9%		
EXPERIMENT	TITER BEFORE	TITER AFTER	PRODUCTION	TITER BEFORE	TITER AFTER	PRODUCTION
1.	1:6	1:48	7×	1:12	1:48	3×
2.	1:6	1:64	10×	1:4	1:32	7×
3.	1:4	1:64	15×	1:2	1:8	3×
4.	1:2	1:16	7×	1:12	1:64	4×
5.	1:8	1:8	-	1:4	1:4	-
6.	1:2	1:32	15×	1:4	1:24	5×
7.	1:8	1:64	7×	1:4	1:8	1×
	Average		8.7×			3.3×

Here also it can be seen that there is a distinctly higher production of agglutinins in the ACTH animals, with the exception of Experiment 5 where both were alike.

In order not to let ourselves be influenced by any prejudice, we had so arranged some of the tests that the readings of the agglutinations were done without previous knowledge as to which animal had received ACTH and which was the control.

The histologic picture of the spleens on the twentieth day after immunization showed great activity of the lymphoid tissue. Many large follicles were present, all with a large germinal center. These latter consist of 80 per cent large or medium-sized immature lymphoid cells, with strongly basophilic cytoplasm (Fig. 1). The large light-staining nuclei had well-developed nucleoli. There were numerous mitoses of these cells. The perifollicular lymphocyte band was only moderately pronounced. The perivascular lymphoid sheaths also contained many large basophilic lymphoid cells showing all the intermediate stages of development into plasma cells. The red pulp contained aggregates of plasma

cells, the majority of which were mature ones of the characteristic Marschalko-type (Fig. 2). Interspersed with these, however, were varying numbers of immature cells displaying the transitions from large immature "lymphoid" cells to specifically recognizable plasma cells (Fig. 3). Compared with earlier stages of the immunization process the number of plasma cell aggregates and the number of cells contained in them was smaller, the cell type more mature. From reconstructions of serial sections it appears that plasma cell aggregates are always located around the smallest arterioles emerging from the lymphoid tissue. They have, in fact, a localization similar to that of the lymphocyte sheaths. It appears as though as a result of the immunization process part of the lymphocyte sheaths are being "replaced" by plasma cell aggregates.

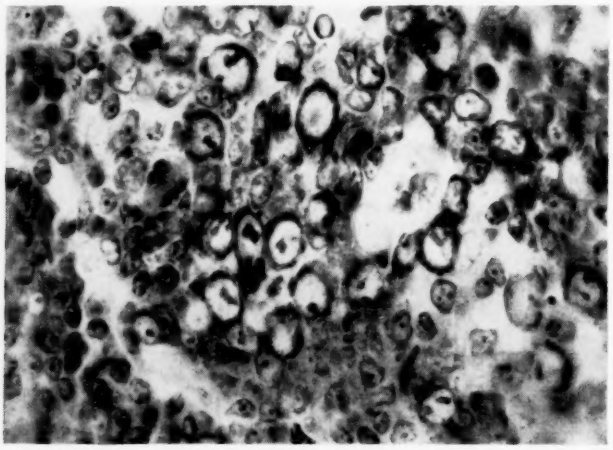

Fig. 1.—Large basophilic lymphoid cells in follicular center of the spleen, twenty-one days after immunization. (Methyl green-pyronin, ×675.)

In view of the enhanced antibody production in ACTH animals, an increase in the percentage of immature plasma cells might be expected. In Experiment 4 the spleen of the ACTH animal, in fact, showed a clear predominance of immature cells in the plasma cell aggregates (Fig. 3), in contrast to the control animal where only mature plasma cells were found (Fig. 2). In most cases it proved very difficult to make reliable estimations of the respective percentages of immature cells in ACTH and control animals.

DISCUSSION

Histologic changes in lymphoid tissue following adrenocortical stimulation have been described by Dougherty and White. They comprised edema, depletion of lymphocytes mitotic inhibition, and the occurrence of macrophages with "tingibele Körper" in the germinal centers—together a picture of lymphoid depression.

Earlier unpublished experiments in this laboratory have demonstrated that from about twelve hours onward after adrenocortical stimulation the picture no longer shows only depression. At this stage resumption of proliferative

activity, particularly in the follicular centers, is found. At twenty-four hours this proliferative activity is clearly above the normal level: in the germinal centers large numbers of immature lymphoid cells are found, many in mitosis. Between them the macrophages with "tingibele Körper" are still visible. Apparently this increased activity following depression can be considered to be a

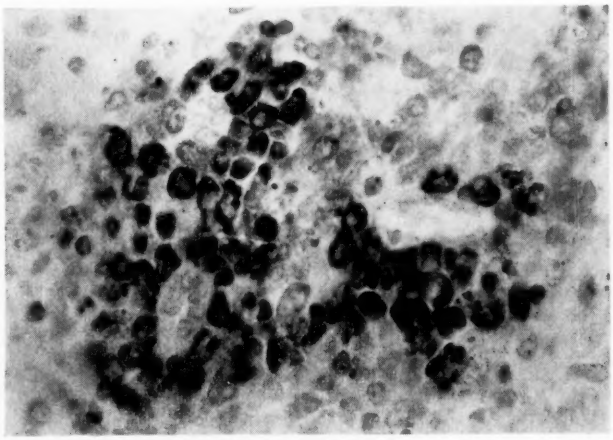

Fig. 2.—Aggregate of mature plasma cells around three capillaries in the red pulp of the spleen of control animal of Experiment 4. (Methyl green-pyronin, ×675.)

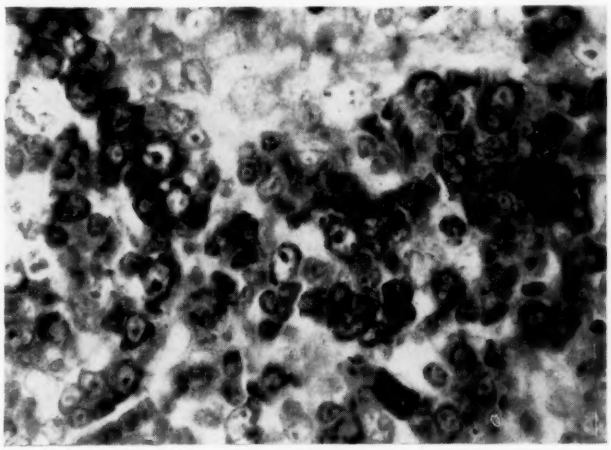

Fig. 3.—Aggregate of immature plasma cells in the red pulp of the spleen of ACTH-animal of Experiment 4. (Methyl green-pyronin, ×675.)

"rebound-phenomenon." The reported increase of the antibody formation twenty-four hours after adrenocortical stimulation suggests that a similar mechanism is at work there, the reactive stimulation of lymphoid tissue giving rise to increased numbers of immature cells responsible for antibody production. It would seem that such a mechanism might prove to be one of the factors

causing prolonged new formation of antibody. It might also play a role in the well-known rise of antibody titer as, e.g., antityphoid agglutinin by an unrelated infectious disease like leptospirosis.

It should be noted that the experimental results reported here were obtained after a single ACTH injection. They do not contradict the observation that pronounced lymphoid atrophy such as is found during prolonged treatment with high doses of ACTH or cortisone may severely impair antibody formation.

The tendency of blood serum titers to fall during the first twenty-four hours after ACTH administration does not, in all probability, depend primarily on decreased antibody formation. It is well known that adrenocortical stimulation brings about protein catabolism—as evidenced by nitrogen balance studies, etc. Serum gamma globulin seems to be involved in this catabolism. One should therefore be very cautious before using serum antibody titers as the sole indication of antibody production. Information obtained from tissue culture experiments appears to be indispensable in many conditions.

SUMMARY

Rabbits were immunized with paratyphoid B-vaccine. Nine days and six months, respectively, after the last antigen injection the rabbits received 10 mg. of ACTH. During the twenty-four hours after this injection the agglutinin titers were followed. There seems to be a tendency to decrease the antibody titers.

Another group of rabbits received 10 mg. of ACTH twenty days after the last antigen injection. A control group received 1 c.c. of 0.9 per cent NaCl. After twenty-four hours fragments of red and white pulp of the spleens were cultivated. The spleens of the rabbits treated with ACTH produced more antibodies than the controls. The histologic picture of the spleens is described. The authors suggested the mechanism of a rebound in the function of the splenic pulp as the cause for the greater antibody production after ACTH.

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ENDOCRINE CHANGES AFTER HEMIADRENALECTOMY AND TOTAL BODY IRRADIATION IN PARABIOTIC RATS

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IN A search for effects of circulating substances following irradiation a large series of parabiotic rats was prepared,[1] which incidentally provided material for an analysis of the pathologic effects of hormonal alterations and interrelations. No morphologic evidence was obtained that any tissue damage produced in one parabiotic rat partner by x-irradiation was transmitted to its nonirradiated shielded partner by substances circulating and crossing the vascular anastomosis. However, there was an interchange of substances with other properties to be described.

MATERIALS AND METHODS

Methods employed have been reported previously in detail.[1] In brief, 292 young Slonaker rats were paired by sex and weight, and joined in parabiosis by modified Bunster-Meyer celioanastomosis. Successfully joined pairs were found to have a vascular interchange of the order of 0.6 per cent of blood volume per minute, similar to that reported by Huff, Troutman, and Van Dyke.[2]

X-irradiation of the right-hand members of healthy pairs was performed twelve to twenty-five days after the juncture, a single total body dose of 400, 800, 1,200, or 1,600 r. The left-hand partners were carefully protected by shielding from radiation exposure. Following irradiation, pairs were sacrificed at intervals from one hour to twenty-nine days after 400 r, one hour to twenty-one days after 800 r, twelve hours to eight days after 1,200 r, and one hour to four days after 1,600 r, complete autopsies performed, and many tissues examined carefully microscopically, particularly for endocrine and radiation reactions.

In 200 additional parabiont pairs from five to twelve days before irradiation, either partner had both adrenals removed surgically. Tissues were examined from 48 normal and 74 adrenalectomized irradiated parabiont pairs. Single and parabiotic control animals were also studied with and without irradiation, respectively.

RESULTS

The endocrine effects observed were chiefly cytologic or histologic alterations, at times supported by findings with special staining or histochemical procedures. Physiologic or assay tests were not made. Factors of importance about which little information was available included the comparative rates of production and utilization of various hormones in normal and irradiated rats.

Adrenal and Effects of Adrenalectomy.—In parabiotic rats with the normal complement of adrenal glands, the endocrine effects of irradiation were not sufficiently striking to be stressed. Their significance became evident, however,

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by comparison of the incidence of end-organ alterations in the hemiadrenalectomized parabiont groups with the effects observed in normal pairs (Table I).

TABLE I. TISSUE CHANGES IN PARABIOTIC RATS, ATTRIBUTED TO ADRENAL CORTICAL FUNCTIONAL ALTERATIONS POSTIRRADIATION

	PROTECTED PARABIONTS		
HYPERPLASIA:	NORMAL	PARTNER ADRENALECTOMIZED	ADRENALECTOMIZED
Thymus	4/12 (33%)	9/25 (36%)	26/31 (84%)
Bone marrow	9/15 (60%)	19/26 (73%)	29/32 (91%)
Interstitial cells	0/9	6/13 (46%)	14/17 (83%)

1. Thymic lymphoid hyperplasia was found in the protected partners in 4 of 12 irradiated normal pairs (33 per cent), up to five days after 800 to 1,600 r. If the irradiated parabiont was also adrenalectomized, the comparable incidence was 9 of 25 (36 per cent), and if the protected rat was adrenalectomized 26 of 31 (84 per cent). In other words, if the rat partner possessing the pair's only adrenal glands was irradiated, the incidence of subsequent thymic hyperplasia in protected rats more than doubled. Thymic hyperplasia was not usual following adrenalectomy alone, and occurred in only 1 of 12 pertinent pairs. Lymph node hyperplasias showed no comparable differences. This appeared to be explained by the high incidence in protected parabionts of hyperplastic lymph nodes after hemiadrenalectomy, regardless of which rat had been irradiated. Thymic tissue, perhaps being less labile in responding to hypoadrenal states, thus demonstrated more often that irradiation had decreased adrenal cortical function.[3]

Alterations of thymic reticulum, particularly hyperplasia, preceded and accompanied the lymphocytic hyperplasia. In irradiated partners, polymorphonuclear leukocytes occasionally infiltrated the Hassall's bodies.

2. Postirradiation bone marrow hyperplasia, involving chiefly leukocyte precursors, was very common in protected rats of hemiadrenalectomized pairs when the animal with adrenals was irradiated, 29 of 32 (91 per cent). It occurred at all time intervals studied following 400 to 1,600 r. In the converse hemiadrenalectomized parabionts, where the remaining adrenals were shielded, 19 of 26 (73 per cent) developed marrow hyperplasia in protected partners. This was similar to the occurrence in comparable normal pairs, 9 of 15 (60 per cent), so that marrow hyperplasia was commonly incidental to parabiosis alone.

At 800 or 1,200 r, mean postirradiation leukocyte counts in the protected partners differed, depending upon which parabiont had been adrenalectomized. This difference was not apparent at 400 or 1,600 r. A leukopenia was observed for five days postirradiation in protected partners which had been adrenalectomized; no extended leukopenia followed when the adrenalectomized rat had also been irradiated. Mean leukocyte counts in the irradiated parabionts showed no differences (Fig. 1).

No evidences of excessive leukocyte sequestration were observed. Evidently the maturation and mobilization of leukocytes were slowed in the postirradiation hypoadrenal state, even when direct effects of irradiation were not involved.[4]

The adrenal margin of safety must be considerable in rats, since such effects became evident only when the one pair of adrenal glands serving two rats was irradiated.

3. Testicular interstitial cell hyperplasia was not found following normal parabiosis, but was seen in one or both parabionts after hemiadrenalectomy and irradiation (Fig. 2, *A* and *B*). In all, it occurred in 22 of 30 adrenalectomized partners (73 per cent) and in 7 of their 30 contralateral rats (23 per cent) throughout the time intervals and radiation dosages studied. Analyzing this same group, when the rat with adrenals was irradiated 14 of 17 protected partners had interstitial cell hyperplasia (83 per cent). When the protected

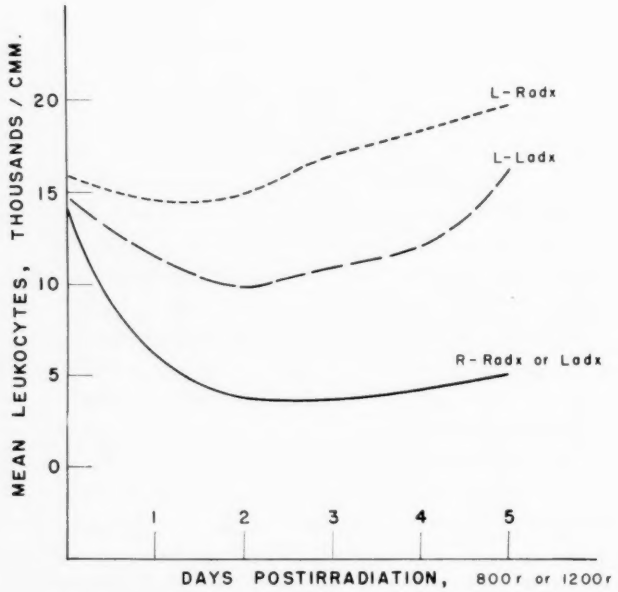

Fig. 1.—Graph demonstrating the lack of mean leukocyte-count-response post irradiation in protected parabionts whose partners were adrenalectomized and irradiated (L-Radx), compared to protected adrenalectomized parabionts (L-Ladx), or irradiated rats of hemiadrenalectomized pairs (R-Radx or Ladx).

rat had the adrenals, 6 of these 13 showed postirradiation interstitial cell overgrowth (46 per cent). In comparable groups of female parabionts, a similar change in ovarian hilus cells was noted, but not regularly enough to determine its frequency. No morphologic effect of adrenal medullary removal was identified.

Testicular tubular changes in irradiated parabionts were identical with exposed single controls and with previous reports, such as those of Fogg and Cowing.[5] Protected partners showed an unusual tubular dystrophy from 1 to about ten days postirradiation in 25 of 39 animals (64 per cent). Sperm maturation ceased at the secondary spermatocyte or spermatid stages. Immature spermatogenic cells, particularly spermatocytes, but also to a lesser extent spermatogonia and spermatids, were observed to be swollen. An abnormally

increased quantity of cytoplasm was present, which was often desquamated free into tubular lumens. Nuclei were normal, or less often multinucleated cells formed, or mitoses and meioses appeared sticky. The changes were not affected by hemiadrenalectomy, and were not found in protected partners beyond fourteen days postirradiation.

Changes in adrenal cortical histology after prior contralateral hemiadrenalectomy were slight but definite. The remaining adrenal glands after hemiadrenalectomy were normally enlarged by cortical hyperplasia, with hypertrophy of cells, particularly in the zona fasciculata, and abundant vacuolated

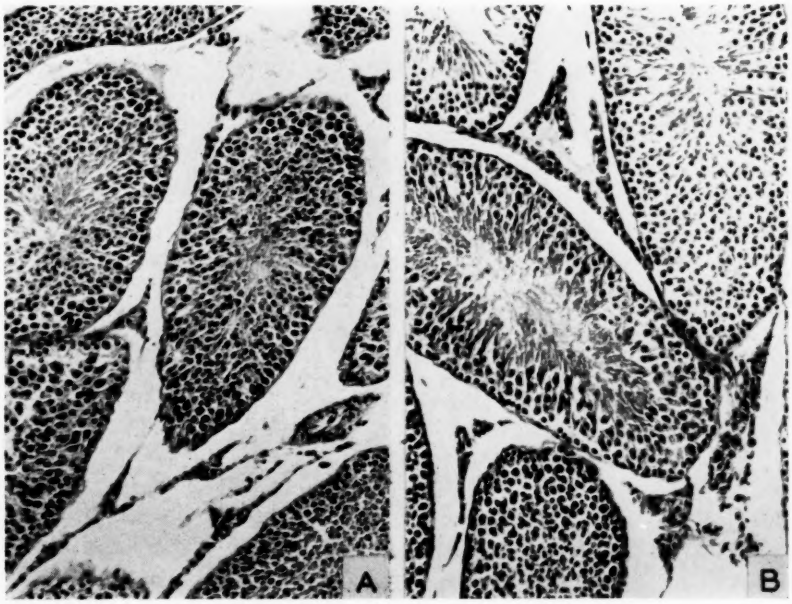

Fig. 2.—Testes of a pair with adrenalectomy and 1,600 r x-radiation of the right partner two days before sacrifice. *A*, Right parabiont's testis, with tubular radiation changes, and normal interstitial cell groups. *B*, Left partner with enlarged spermatogonia and spermatocytes. Interstitial cells increased. (Hematoxylin and eosin, ×125.)

lipoid content. Post irradiation no alterations were observed if the remaining adrenals were in the protected partners. In irradiated parabionts' adrenals postirradiation, when the protected partners were adrenalectomized, there was a loss of finely vacuolated lipoid from cortical cells for four to five days. At this time, Ashbel-Seligman stain for free carbonyl groups,[6] criticized by Gomori,[7] showed decreased purple coloration of the cortices, compared to staining of single irradiated control rats or protected adrenals of hemiadrenalectomized pairs (Fig. 3, *A* and *B*).

Hemiadrenalectomized parabionts developed glandular fat, or accessory adrenal tissue, in the retroperitoneal, mediastinal, and cervical connective tissues. This process and its cytology were not noticeably altered by irradiation.

TABLE II. TISSUE CHANGES IN PARABIOTIC RATS, ATTRIBUTED TO ANTERIOR PITUITARY FUNCTIONAL ALTERATIONS POSTIRRADIATION

	PARABIONT RATS					
	NORMAL		IRRADIATED RAT ADRENALECTOMIZED		PROTECTED RAT ADRENALECTOMIZED	
	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT
Ovary						
Granulosa cell hyperplasia	----	6/8 (75%)	----	10/12 (83%)	----	**13/17 (77%)**
Luteinized follicles	0/14	1/16 (6%)	2/9 (22%)	5/11 (46%)	2/14 (14%)	1/16 (6%)
Pancreas						
Islet hyperplasia	1/9 (11%)	0/9	5/19 (25%)	1/9 (11%)	**6/19** (32%)	**1/11** (9%)
Islet atrophy	0/12	0/12	1/10 (10%)	2/9 (22%)	3/20 (15%)	**4/23** (17%)
Depleted acinar basophilia	1/34 (3%)	2/34 (6%)	10/23 (44%)	6/23 (26%)	10/28 (36%)	**1/31** (3%)
Duct dystrophy	0/34	0/34	5/23 (22%)	0/23	3/28 (9%)	**1/31** (3%)
Thyroid						
Hyperplasia	2/12 (16%)	1/13 (8%)	11/26 (42%)	15/20 (75%)	6/23 (26%)	**16/29** (55%)

Pituitary.—As in the adrenal gland, end-organ effects of pituitary stimulation or its lack were more easily established than changes in the gland's histology or cytology (Table II).

Adrenocorticotropin.—Since the adrenal cortex was either normal or hyperplastic, and the lipoid content intact or depleted post irradiation, the evidence pointed to normal or increased ACTH production post irradiation. Other tissues such as salivary gland and pancreas suggested by an early postirradiation loss of cytoplasmic basophilia an increased ACTH secretion, at least in the irradiated partners of hemiadrenalectomized pairs.

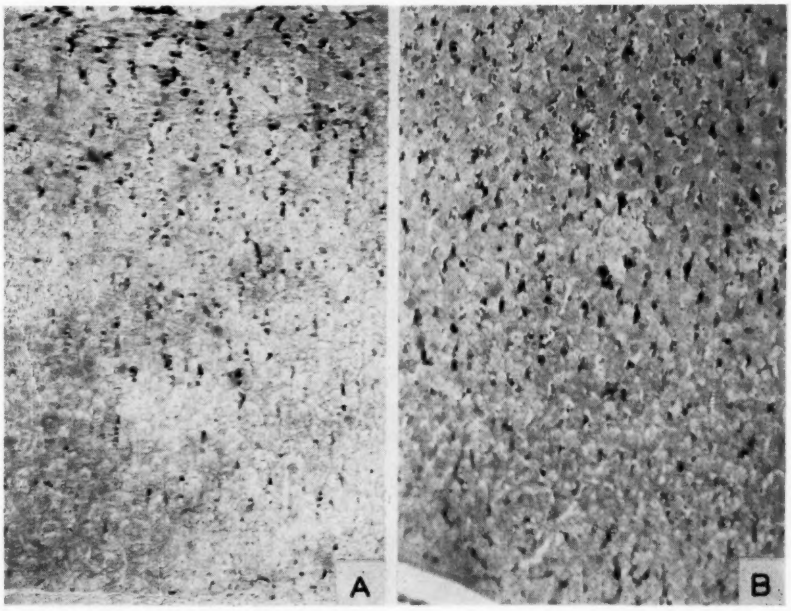

Fig. 3.—Comparison of Ashbel-Seligman stains for free carbonyl groups. In *A* the irradiated adrenal cortex is shown four days after 400 r, partner adrenalectomized. *B*, half thickness of the hyperplastic adrenal cortex of a protected parabiont, partner adrenalectomized and irradiated with 800 r, one day before. The darker gray shade of this adrenal cortical tissue is comparable with normal controls, but the color is depleted in the irradiated adrenal. The small black objects are red blood cells in sinusoids. (×125.)

Gonadotropins.—No morphologic evidence of reduction of gonadotropin secretion was evident in either sex for the relatively short periods of observation following total body irradiation of one parabiont, up to the level of 1,600 r. There was no additive effect of hemiadrenalectomy. Hyperplasias of ovarian granulosal cells or testicular spermatocytes in protected partners post irradiation were considered responses to decreased total gonadal hormone production and release of pituitary inhibition.

Growth Hormone.—A proliferative increase of pancreatic islet tissue (Fig. 4) was found in a minority of irradiated rats whose partners had been adrenalectomized, 6 of 19 (32 per cent), at all dosage levels, one to five days post irradiation. It was next most frequent in irradiated rats previously adrenalectomized, 5 of 19 (25 per cent). In protected rats with or without adrenals

this islet change was 10 per cent, 2 of 20. Decreased islet tissue was not identified with the same certainty as islet hypertrophy and hyperplasia.

Those animals most frequently showing islet stimulation were in post-irradiation hypoadrenal states, the remaining adrenals having been placed experimentally under maximum strain. This indicated indirectly that the pituitary growth hormonal effects[8, 9] had been either unaffected or stimulated post irradiation.

Cytoplasmic basophilia of pancreatic acinar cells was sometimes depleted post irradiation, in either or both partners. The depletion occurred more often in hemiadrenalectomized pairs, one to five days after 400 to 1,600 r. Ducts showed concomitant degenerative alterations, with necrobiosis and desquamation in a few irradiated animals. Dilated ducts containing inspissated eosinophilic material were found rather commonly in irradiated partners of hemiadrenalectomized pairs. The changes were similar to those described in human material by Baggenstoss.[10]

The only somatic hyperplasia frequently identified, aside from endocrine glands or end-organs, lymphoid and hematopoietic tissues, was in small intestinal epithelium. Post irradiation, one to five days after 400 to 1,600 r, protected partners showed this hyperplasia in 2 of 14 normal pairs (14 per cent), in 13 of 26 protected rats whose partners were adrenalectomized and irradiated (50 per cent), and in 16 of 33 protected adrenalectomized parabionts (49 per cent). It seemed likely that it was the local interplay of circulating synergistic (intestinal epithelial breakdown products?) or antagonistic (adrenal cortical steroids?) factors which determined where and whether GH produced local growth or not.

TABLE III. PITUITARY COUNTS, POSTIRRADIATION

		CHROMOPHOBES RANGE; MEAN	EOSINOPHILS RANGE; MEAN	BASOPHILS RANGE; MEAN
Normal[11]		48-53%	42-45%	4- 6%
1 to 3 days postirradiation				
4 controls		43-55% (48%)	33-46% (42%)	10-12% (11%)
5 normal parabionts	R	48-80 (62)	16-50 (33)	2- 8 (5)
	L	42-60 (50)	34-52 (43)	5-10 (7)
7 pairs, irradiated	R	31-69 (48)	23-54 (41)	4-20 (10)
rat adrenalectomized	L	35-68 (47)	24-53 (38)	8-26 (15)
9 pairs, protected	R	41-60 (50)	32-44 (38)	7-19 (12)
rat adrenalectomized	L	48-60 (54)	28-38 (33)	10-17 (13)
4 to 7 days postirradiation				
2 controls		52-59% (56%)	33-35% (34%)	6-15% (11%)
3 normal parabionts	R	51-59 (55)	31-38 (35)	6-15 (10)
	L	37-63 (53)	30-45 (35)	7-18 (12)
5 pairs, irradiated	R	43-53 (49)	28-36 (33)	13-22 (18)
rat adrenalectomized	L	37-50 (43)	37-53 (44)	6-21 (13)
6 pairs, protected	R	44-58 (51)	26-46 (36)	10-18 (14)
rat adrenalectomized	L	46-58 (52)	28-41 (35)	8-17 (13)
8 to 29 days postirradiation				
3 controls		43-45% (44%)	39-41% (40%)	14-17% (16%)
10 normal parabionts	R	44-60 (52)	29-46 (37)	6-25 (11)
	L	35-70 (57)	24-45 (34)	3-20 (9)
3 pairs, either	R	31-46 (39)	39-47 (44)	13-22 (17)
rat adrenalectomized	L	40-45 (42)	45-46 (46)	10-14 (13)

Thyrotropin.—Postirradiation thyrotropic stimulatory effects were evident practically only in hemiadrenalectomized parabionts. Protected partners, either adrenalectomized, 16 of 29 (55 per cent), or the irradiated partner having been adrenalectomized, 15 of 23 (75 per cent), had microscopic thyroid hyperplasia. Follicular epithelium was tall cuboidal to columnar, with reduction and peripheral vacuolization of colloid (Fig. 5). Irradiated adrenalectomized partners had this change in 11 of 26 (42 per cent). The hyperplasia was most often observed in protected partners, particularly those whose partners had been adrenalectomized, and was found at all the radiation doses employed over the complete range of time intervals studied. In irradiated parabionts only exceptionally a few follicular cells were degenerated, suggesting slight irradiation effect.

Fig. 4.—Increased size and abundance of pancreatic islets, five days after irradiation with 1,600 r, protected parabiont adrenalectomized. (Hematoxylin and eosin, ×50.)

Fig. 5.—Thyroid hyperplasia, two days after 1,200 r x-radiation to adrenalectomized partner. (Hematoxylin and eosin, ×125.)

Cytology.—Investigation of pituitary cytology of the anterior lobe was carried out by cell counts of 109 rat glands stained with the Mallory aniline blue method. Whenever possible, at least four high-power fields were completely counted, up to 1,600 cells. Because of postmortem changes and capricious staining in some glands, the per cent figures were not considered absolute values (Table III).

Direct cytologic effects of irradiation were particularly observed after 1,600 r in chromophobe cells, whose nuclei became swollen with loss of chromatin. Also, eosinophils were occasionally shrunken and basophils degranulated in preparations made one to three days post irradiation. From three to seven

days post irradiation, chromophobe nuclei were enlarged with prominent nucleoli present in increased numbers. Eosinophils and basophils were observed to be hypertrophied. Simultaneously, the cellular and nuclear hypertrophies could also be observed in the protected parabionts. These changes were more striking in both groups of hemiadrenalectomized parabionts than in either normal parabionts or controls.

Analysis of cell counts (Table III) suggested a postirradiation hyperplasia of basophils in hemiadrenalectomized pairs from a level of about 8 per cent to as much as 20 per cent, without regard to which animal was adrenalectomized, irradiated, or the sex involved. It was most evident at five to fifteen days post irradiation. A hyperplasia of eosinophils occurred exceptionally in either partner, within the same time interval. The least labile group of pituitaries observed was in pairs where the protected partners were adrenalectomized.

Ovary.—Associated with typical radiation damage with destruction of ova and the granulosa cells of developing follicles in exposed partners, protected parabionts showed after one to five days a hyperplasia of granulosa cells in 29 of 37 rats (79 per cent). Masses of these cells partly filled follicular cavities, or proliferated in the interstitial stroma. By three to five days post irradiation, the exposed granulosa cells began to show regenerative hyperplasia. With 400 r the ovarian architecture was restored to normal by the fourteenth day post-irradiation, but degeneration and regeneration were evident beyond the third week after 800 r.

Primordial ova and their granulosa cells were more resistant to x-radiation, and corpora lutea did not appear affected. As mentioned, normal or adrenalectomized pairs occasionally had hyperplasias of ovarian hilar cells, without regularity.

Hemiadrenalectomized pairs showed no significant differences from normal parabionts in their ovarian reactions, except that occasional ovaries had also a luteinization of unruptured follicles. The luteinized follicles appeared more often in the partner with adrenals, whether protected or irradiated (Table II).

Parathyroid—No changes associated with irradiation or hemiadrenalectomy were observed. Occasionally slight swelling or degeneration of a few cells was observed, adjacent to acute and chronic tracheitis.

Salivary Gland.—Sexual dimorphism characterizes the eosinophilic duct cells of rodents, in that males have apical cytoplasmic granules which females lack.[12] Post irradiation in both partners these duct cells frequently showed marked degenerative changes.

Apical cytoplasmic granules were depleted in males, both irradiated and protected, but never completely disappeared. Females in six instances post irradiation, 400 to 1,600 r, showed masculine-type apical epithelial granules. They occurred simultaneously in both partners of normal and hemiadrenalectomized pairs, within six days post irradiation. It was not proved that this represented masculinization, since the ducts were undergoing rather brisk degenerative and regenerative alterations concomitantly.

DISCUSSION

The localizations of radiation reactions in the exposed parabiont rats were identical with those in single animals. Sites of rapid nucleoprotein synthesis and mitotic activity suffered maximum damage, as observed microscopically in lymphoid and hematopoietic tissues, small intestinal epithelium, and gonads. The general body reactions which followed involved most endocrine glands, but there was nothing unique, peculiar, or disproportionate in the types and degrees of endocrine alterations observed. Alone, no endocrine function appeared to determine survival. It was the amount of direct tissue damage by irradiation which mattered most in this regard.

Of all endocrine alterations in parabiotic rats, those of gonadal tissues were most marked. Granulosa-cell necrosis in irradiated ovaries stimulated in protected partners a hyperplasia of the same cells, which responded faster than the damaged ovaries could regenerate. Decreased inhibition of pituitary gonadotropins, particularly FSH, was inferred. A hyperplasia of anterior pituitary basophils could be correlated morphologically. Evidence for increased LH production was adduced in only about one-half of protected rats whose partners were both adrenalectomized and irradiated, and in a scattering of other parabionts.

A testicular response was found which supported belief in a tubular-ripening-hormone.[12, 13] Consideration separately at greater length is planned. Hormones like FSH and LH appeared operative also in males. Testicular interstitial cell hyperplasia stimulated by pituitary LH occurred only in hemiadrenalectomized rats.

Adrenal cortical reactions were investigated. In normal parabionts the margin of safety of adrenal capacity appeared to be effective in preventing any morphologic alterations post irradiation. After contralateral hemiadrenalectomy, irradiated adrenal cortex had demonstrable cytologic loss of lipid and free carbonyl groups. End-organ changes such as thymic, hematopoietic, and testicular interstitial cell hyperplasias pointed to a temporary loss of effective adrenal cortical control. No differences in the radiation sensitivity of different adrenal functions were observed.

Indirect adrenal relationships were evident in the pancreatic and thyroid alterations. Hemiadrenalectomized pairs showed pancreatic islet hyperplasia most often in the irradiated rat. This could be interpreted as indicating normal or increased pituitary pancreatropic secretion (growth hormone), radiation insensitivity of islet tissue, and weakening of adrenal inhibition after hemiadrenalectomy. Thyroid hyperplasia was also produced more often in hemiadrenalectomized pairs. Protected parabionts were most often affected in animals with relative hypoadrenal function.

From the end-organ changes there was no evidence that total body irradiation to one parabiont in the range 400 to 1,600 r reduced the secretion of any anterior pituitary lobe hormone. Adrenocorticotropic, pancreatropic, growth hormone, and thyrotropic effects were all evident. They were brought out more clearly by previous adrenalectomy of one parabiont and conditions of strain

upon the remaining adrenal tissue. It was not certain that any pituitary hormone secretion was increased, aside from the expected pituitary preponderance in a rat pair with two pituitary glands and only one set of adrenals. The suggestive evidence was that post irradiation at least the secretion of growth hormone was increased. A few cytologic changes in irradiated rats pointed to direct pituitary radiation damage with 1,600 r, but without any consequent reduction of function being evident.

It was interesting to find that tissue responses in endocrine glands were dependent as much on decreased inhibition, such as that following hemiadrenalectomy, as on pituitary activity. Also, tissue destruction in the partner appeared frequently to be a necessary preliminary to a hyperplastic response. In most organs, including intestine and gonads, the partner's tissue necrobiosis localized the growth stimuli to these organs. However, pancreatic islets and thyroid responded to stimulation following damage to other tissues, and without evident antecedent local damage. To this extent, the belief of Smith[14] that tissue necrosis is a nonspecific pituitary stimulus was supported.

Direct effects of irradiation upon testicular interstitial cells, pancreatic islets, parathyroid, and thyroid were insignificant.

As in most morphologic studies, physiologic mechanisms and hormonal participation were only inferred. The reactions observed, as in many biological systems, had general patterns. However, there were individual or group exceptions to the usual responses. The parabiotic juncture made a complicated test object in which many changes occurred concomitantly. Practically all the observations were explicable, however, on the basis of present endocrinologic knowledge.

SUMMARY

A morphologic study was made of endocrine alterations in parabiotic rats during short intervals post irradiation. Aside from the radiosensitive gonadal tissues, few endocrine gland changes were commonly observed, except after prior hemiadrenalectomy. In protected animals ovarian granulosa cells showed postirradiation hyperplasia.

In relatively strained adrenal functional states, radiation produced a decrease of lipid and free carbonyl groups in the adrenal cortex. Increased incidences of hyperplasias of thymus, bone marrow leukocyte precursors, and testicular interstitial cells indicated that under maximal experimental strain irradiation had reduced adrenal cortical functions. Pancreatic islets, ovarian theca cells, and thyroid also more often became hyperplastic in postirradiation hypoadrenal conditions. A considerable normal adrenal margin of safety was evident.

Pituitary cells showed slight direct radiation effects, after 1,600 r. Anterior pituitary functions seemed unaffected or stimulated post irradiation, as shown by tissue responses attributed to gonadotropin, adrenocorticotropin, growth hormone, and thyrotropin secretion. Cell counts indicated irregular postirradiation hyperplasias of pituitary basophils and eosinophils.

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THE SIGNIFICANCE OF THE PULMONARY ARTERIAL WEDGE PRESSURE

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THE procedure of right heart catheterization developed by Cournand and Ranges[1] has proved to be an important aid in the diagnosis of cardiovascular disease. Pressures can be recorded, oxygen content of the blood can be determined, and radiopaque material can be instilled through the catheter at selected sites. The procedure has been especially helpful in the study of congenital cardiovascular disease. It also has been used widely in the study of pulmonary disease, and has provided a great deal of information about the physiology of the cardiovascular system and the lungs in health and disease.

Dexter and associates[2] and Gorlin and Gorlin[3] extended the procedure when they wedged the cardiac catheter into small pulmonary arteries and obtained pressure readings which can be employed in calculating pulmonary vascular resistance and the area of the mitral valve.

A pressure recorded through a catheter wedged tightly in a small pulmonary artery has been referred to as a "capillary," "venous," or "wedge" pressure. It is the purpose of this study to determine the significance of pressures obtained through a catheter wedged in a small pulmonary artery.

METHOD

The study was carried out in six patients undergoing thoracotomy for pulmonary disease and four patients with mitral valve disease undergoing mitral commissurotomy (Table I). The patients who underwent surgery for pulmonary disease had no demonstrable cardiovascular disease. Pulmonary arterial wedge pressures and pulmonary arterial trunk pressures were obtained through catheters inserted into branches of the pulmonary artery (Fig. 1). Pulmonary venous wedge pressures and left atrial pressures were obtained through catheters inserted into branches of the pulmonary veins (Fig. 1). The wedge pressures were obtained through No. 6F catheters with an opening in the end. The pulmonary arterial trunk and left atrial pressures were obtained through No. 6F catheters with an opening in the end and two openings at opposite points on the circumference immediately proximal to the end. Pressures were recorded while the chest was open. The wedge pressures were obtained in portions of the lung which were aerated and grossly normal. The superior surface of the presenting atrium was taken as the reference level from which pressures were measured. The pressure tracings were calibrated with a mercury manometer. The arterial wedge and left atrial pressures were made simultaneously or within a few minutes of each other as were the venous wedge and pulmonary arterial trunk pressures. A Sanborn four-channel amplifier and Statham strain gauges were used.

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TABLE I

PATIENT	AGE	DIAGNOSIS	MEAN PULMONARY ARTERIAL WEDGE PRESSURE (MM. HG)	MEAN LEFT ATRIAL PRESSURE (MM. HG)	MEAN PULMONARY VENOUS WEDGE PRESSURE (MM. HG)	MEAN PULMONARY ARTERIAL TRUNK PRESSURE (MM. HG)
1	20	Benign pulmonary cyst	6.0	11.7	22.6	18.0
2	41	Unresolved pneumonia	13.9	16.1	13.6	20.9
3	59	Pulmonary tuberculosis	5.8	11.4	19.2	23.9
4	44	Pulmonary infiltration of undetermined etiology	--	--	33.1	35.8
5	53	Bronchogenic carcinoma	--	--	35.9	32.1
6	28	Pulmonary tuberculosis	16.4	16.0	22.2	24.7
7	40	Mitral valve disease with atrial fibrillation	36.8	33.5	52.0	46.7
			37.4*	32.1*	--	--
8	60	Mitral valve disease with atrial fibrillation	17.3*	14.1*	49.8*	51.5*
9	34	Mitral valve disease with atrial fibrillation	23.8	27.9	--	--
			21.0*	23.7*	--	--
10	36	Mitral valve disease	27.3	26.6	67.3	65.7
			22.2*	27.3*	44.8*	50.8*

*After commissurotomy.

RESULTS

The mean pressures obtained in the ten patients studied are recorded in Table I. An elevated pulmonary arterial trunk pressure was recorded for patient No. 4 and patient No. 5. An explanation for the elevated pressure in these cases cannot be given. An elevated pulmonary arterial trunk pressure was obtained for all patients who had mitral valve disease. The left atrial pressure was elevated in these patients except in the case of patient No. 8. This patient had been less incapacitated by his disease than patient No. 7 and patient No. 9 who also exhibited atrial fibrillation.

Pressure tracings are shown in Fig. 2, Fig. 3, and Fig. 4, which indicate that fluctuations in the left atrial pressure are not reflected accurately in the arterial wedge tracing. Pressure tracings are shown in Fig. 2 and Fig. 3 which indicate that the degree of mitral regurgitation cannot be predicted from inspection of the arterial wedge tracing. Pressure tracings are shown in Fig. 5 which indicate that fluctuations in the pulmonary arterial trunk pressure are not reflected quantitatively in the venous wedge tracing. Pressure tracings are shown in Fig. 4 and Fig. 5 which indicate that neither the arterial nor venous wedge tracing exhibits characteristics which distinguish one from the other. Respiratory artifacts are present.

The mean pulmonary arterial wedge and mean left atrial pressures shown in Table I are plotted in Fig. 6. The mean pulmonary venous wedge and mean pulmonary arterial trunk pressures shown in Table I are plotted in Fig. 7. Pressures were obtained before and after commissurotomy in patients with mitral valve disease. Pressures obtained after commissurotomy were not treated statistically except in the case of patient No. 8 whose pressures were not recorded before commissurotomy.

The correlation coefficient between the left atrial and pulmonary arterial wedge pressures is 0.9505; p is less than 0.01. The correlation coefficient between the pulmonary arterial trunk and venous wedge pressures is 0.9721; p is

less than 0.01. There is no significant difference between the means of the left atrial and pulmonary arterial wedge pressures; t is 0.2613; p is greater than 0.8. There is no significant difference between the means of the pulmonary arterial trunk and pulmonary venous wedge pressures; t is 0.05; p is greater than 0.9.

There is no significant difference from zero of the mean difference between the left atrial and pulmonary arterial wedge pressures; t is 0.9286; p is greater than 0.3. There is no significant difference from zero of the mean difference between the pulmonary arterial trunk and pulmonary venous wedge pressures; t is 0.66; p is greater than 0.5.

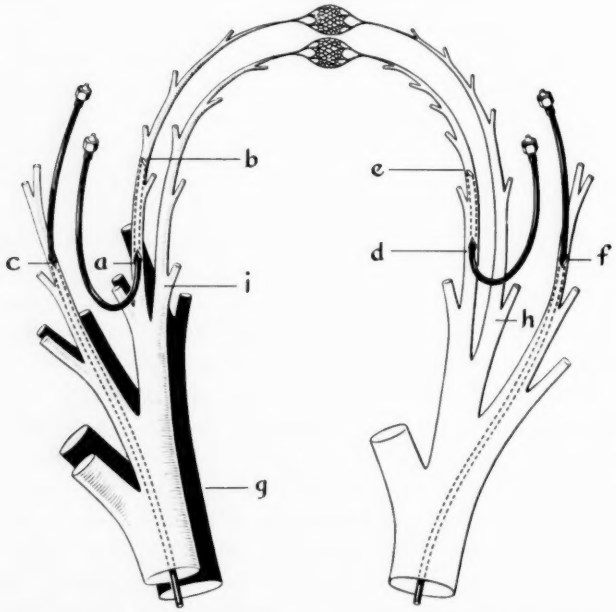

Fig. 1.—A semischematic representation of a portion of a lobule of the lung showing catheters in position. *a*, Catheter inserted into a branch of the pulmonary artery and wedged at *b*; *c*, catheter inserted into a branch of the pulmonary artery and extended into the pulmonary arterial trunk; *d*, catheter inserted into a branch of the pulmonary vein and wedged at *e*; *f*, catheter inserted into a branch of the pulmonary vein and extended into the left atrium; *g*, bronchus; *h*, the pressure in this compartment is recorded through a catheter wedged at *b*; *i*, the pressure in this compartment is recorded through a catheter wedged at *e*.

DISCUSSION

The data presented indicate that the pulmonary arterial wedge pressure is equal to the left atrial pressure. This relationship has been reported previously. Dow[4] introduced catheters via a jugular vein and a femoral artery and found the difference between the arterial wedge and left atrial pressures to be negligible. Ankeney[5] introduced catheters into branches of the pulmonary artery and vein and obtained arterial wedge pressures which corresponded to pressures obtained in the left atrium. Both of these studies were carried out in dogs.

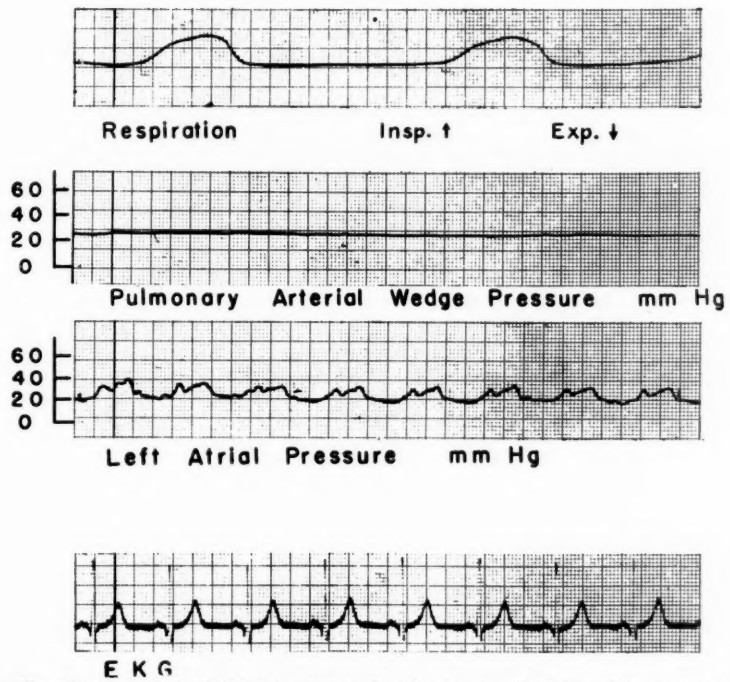

Fig. 2.—Simultaneous left atrial pressure and pulmonary arterial wedge pressure before commissurotomy (No. 10, Table I).

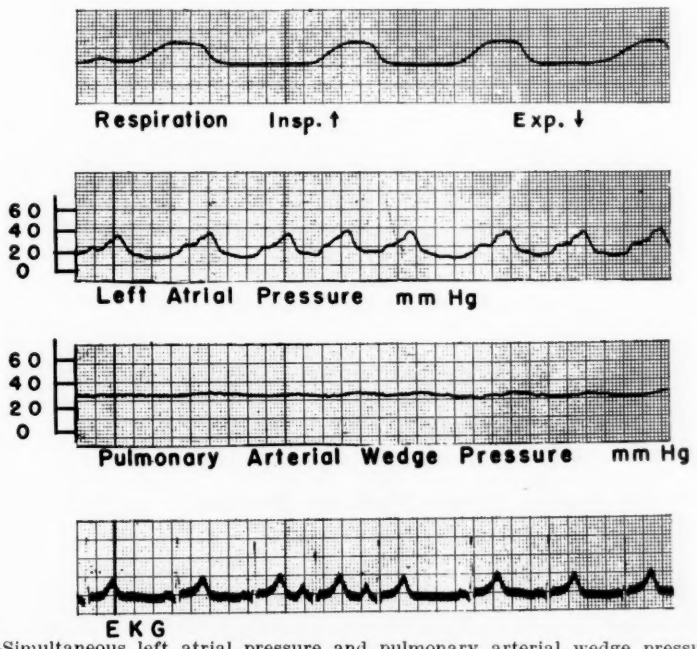

Fig. 3.—Simultaneous left atrial pressure and pulmonary arterial wedge pressure after commissurotomy (No. 10, Table I).

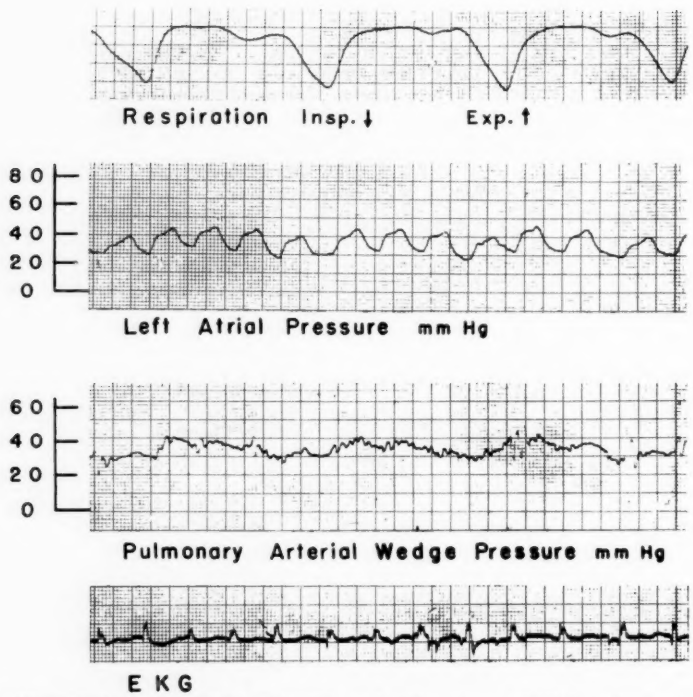

Fig. 4.—Simultaneous left atrial pressure and pulmonary arterial wedge pressure after commissurotomy (No. 7, Table I).

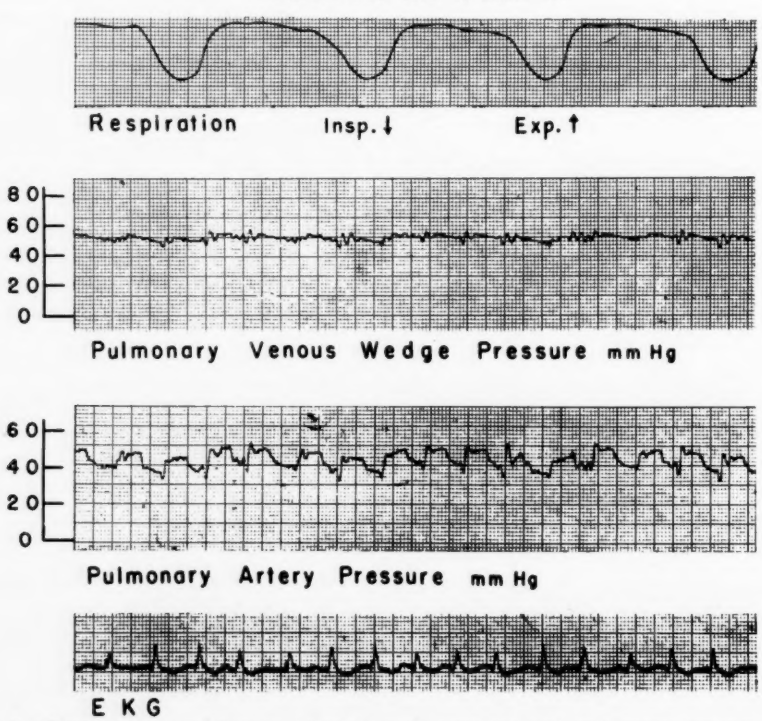

Fig. 5.—Simultaneous pulmonary arterial trunk pressure and pulmonary venous wedge pressure after commissurotomy (No. 7, Table I).

Calazel and co-workers[6] reported close agreement between the arterial wedge and left atrial pressures in patients with an interatrial septal defect whose left atrial pressure was 10 mm. Hg or less. In three patients whose left atrial pressure was greater than 10 mm. Hg, the arterial wedge pressure was elevated.

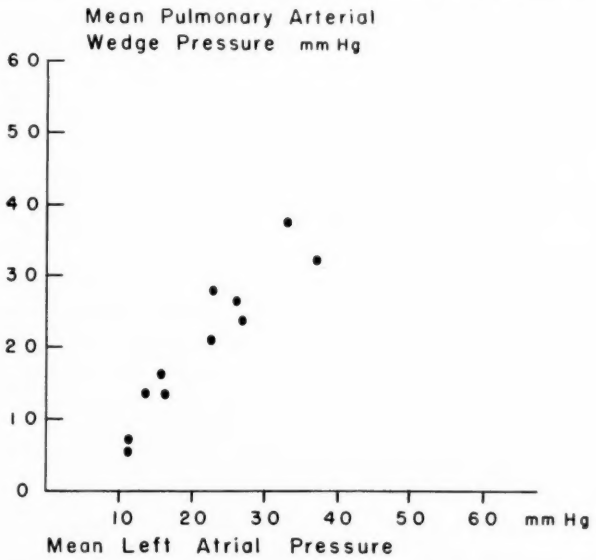

Fig. 6.—Relationship of the mean left atrial pressure to the mean pulmonary arterial wedge pressure.

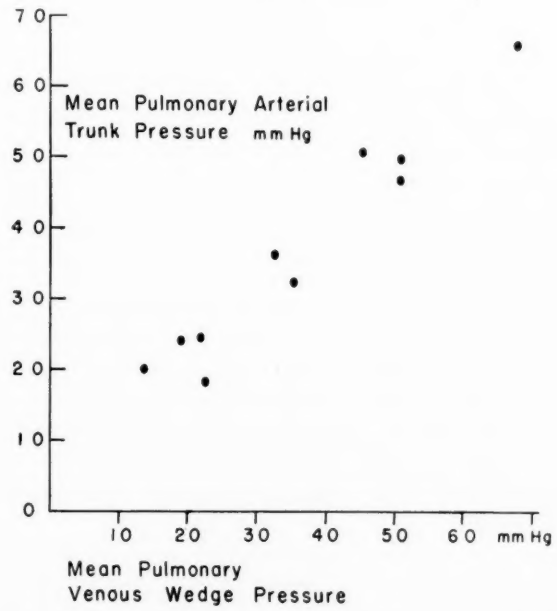

Fig. 7.—Relationship of the mean pulmonary venous wedge pressure to the mean pulmonary arterial trunk pressure.

In one case the mean pulmonary arterial pressure was 23, the mean wedge pressure was 14, and the mean left atrial pressure was 11 mm. Hg. In another case the mean pulmonary arterial pressure was 43, the mean wedge pressure was 15, and the mean left atrial pressure was 12 mm. Hg. In a third case the mean pulmonary arterial pressure was 21, the mean wedge pressure was 19, and the mean left atrial pressure was 12. It is suggested that the arterial wedge and left atrial pressures in the first two cases were actually in close agreement and that the pressure reported as a wedge pressure in the third case was actually a pulmonary arterial pressure.

Connolly and co-workers[7] studied patients with mitral valve disease who underwent commissurotomy; a close relationship was found between the arterial wedge and left atrial pressures.

The data obtained in the study being reported indicate that the pulmonary venous wedge pressure is equal to the pulmonary arterial trunk pressure. The pulmonary venous wedge pressure has been previously recorded; but the relationship between this pressure and the pulmonary arterial trunk pressure has not been noted.

Hellems and associates[8] introduced a catheter into a jugular vein and into a carotid artery in dogs and obtained wedge pressures simultaneously on both sides of the pulmonary capillary plexus. Hellems and collaborators[9] also reported pulmonary venous wedge pressures in two patients with an interatrial septal defect. In the first case the mean pulmonary arterial pressure was 30, the mean arterial wedge pressure was 10, and the mean venous wedge pressure was 11 mm. Hg. In the second case the mean pulmonary arterial pressure was 20, the mean arterial wedge pressure was 11, and the mean venous wedge pressure was 11 mm. Hg. It is suggested that the pressures recorded as venous wedge pressures in these cases were actually venous pressures.

Calazel and associates[6] obtained wedge pressures in two patients with an interatrial septal defect. In one of these patients the mean pulmonary arterial pressure was 19 and the mean venous wedge pressure was 15 mm. Hg. In the other patient the mean pulmonary arterial pressure and the mean venous wedge pressure was 12 mm. Hg. The relationship between the two pressures in these cases is in agreement with the data presented in this study.

There are certain anatomical features of the pulmonary vasculature[10] which may explain why the arterial and venous wedge pressures correlate with those in the left atrium and arterial trunk: (1) anastomosis does not take place between branches of the pulmonary artery; (2) there are no valves in the vessels of the lesser circulation; (3) the capillary plexus in the wall of the alveolus is exceedingly rich.

When a catheter is wedged in a pulmonary artery or vein, the flow of blood is interrupted in the portion of the lung supplied by the occluded vessel. The lumen of the catheter communicates through the capillary plexus with a volume of blood which extends from a small artery to the mitral valve or from a small vein to the pulmonic valve. The pressure recorded in either position is the pressure in the vascular compartment beyond that in which the flow of blood has been interrupted. The capillary plexus and small arteries and veins serve as an

extension of the catheter into these compartments. The pressure recorded through a catheter wedged in a small artery is undoubtedly the pressure in the small veins (Fig. 1). The pressure recorded through a catheter wedged in a small vein is undoubtedly the pressure in the small arteries (Fig. 1). The wedge pressures correlate with the pressure in the left atrium and the pressure in the arterial trunk because the pressure gradient in the large pulmonary arteries and veins is small with respect to the degree in which a particular pressure measurement may be in error. Neither the arterial nor the venous wedge pressure is a measurement of the pressure in the pulmonary capillaries in which the flow of blood has not been interrupted. The capillary pressure must be determined directly in the capillaries.

The difference between the pulmonary arterial trunk pressure and the pulmonary arterial wedge pressure has been employed in the calculation of pulmonary vascular resistance. The data presented indicate that the difference between these pressures is approximately equal to the pressure gradient across the entire lesser circulation. The pulmonary vascular resistance which is calculated is approximately equal to the total pulmonary vascular resistance. It is evident that pulmonary "arteriolar" resistance cannot be measured if the capillary pressure is not known. Increased resistance in the capillaries may contribute to the increased total pulmonary vascular resistance which has been attributed to increased "arteriolar" resistance.

There has been controversy regarding whether or not cyclic variations can be identified in the pulmonary arterial wedge pressure tracing which are related to those occurring in the left atrial pressure tracing.

Ankeney[5] reported that when a catheter is properly wedged in a pulmonary artery the recorded tracing shows an essentially constant pressure. Wedge pressures in this study were obtained in dogs through catheters inserted directly into a branch of the pulmonary artery. Ankeney stated that cyclic variations in the pulmonary arterial wedge pressure tracing are probably related to changes in pressure in the pulmonary artery rather than to changes in pressure in the left atrium.

Connolly[7] found that the pulmonary arterial wedge pressure tracing was similar to the left atrial pressure tracing in patients with mitral stenosis. Wedge pressures in this study were obtained after thoracotomy through catheters inserted directly into a branch of the pulmonary artery. Connolly was unable to conclude, however, that the pulmonary arterial wedge pressure tracing is a reliable indication of the presence or degree of mitral regurgitation.

The pulmonary arterial and venous wedge pressure tracings obtained in the study being reported did not exhibit fluctuations which could be related consistently to changes in pressure in the left atrium or pulmonary arterial trunk. The suppression of cyclic fluctuation is considered to be due to damping in the pulmonary capillary plexus.

Particular care should be taken in right heart catheterization to obtain an arterial wedge pressure which is accurate if pulmonary resistance or valve areas are to be calculated. The use of catheters with a single opening in the end facilitates the recording of an accurate wedge pressure. An arterial wedge

tracing which shows essentially no fluctuations and which can be duplicated on repeated wedging can be accepted as an accurate wedge pressure. An accurate wedge pressure will usually be obtained if blood which is completely saturated with oxygen can be withdrawn through the catheter. Occasionally blood cannot be withdrawn through a catheter which transmits a pressure which can be duplicated. It is to be noted that an arterial wedge tracing obtained through a catheter which traverses the right heart will show fluctuations due to movement of the catheter which will not be found if the catheter is inserted directly into a branch of the pulmonary artery.

CONCLUSIONS

The pulmonary arterial wedge pressure is approximately equal to the left atrial pressure and is not a measurement of the pressure in capillaries in which the flow of blood has not been interrupted.

The pulmonary venous wedge pressure is approximately equal to the pulmonary arterial trunk pressure and likewise is not a measurement of the pulmonary capillary pressure.

The difference in pressure between the pulmonary arterial trunk and pulmonary arterial wedge pressures is approximately equal to the pressure gradient across the entire lesser circulation and is employed in the calculation of total pulmonary vascular resistance.

The contour of the arterial wedge tracing does not reflect the contour of the left atrial tracing.

Attention must be paid to the proper wedging of the catheter in right heart catheterization.

The authors gratefully acknowledge the advice of Herbert S. Wells, M.D., who guided the course of this study.

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PENICILLIN G AND BENZETHACIL BY MOUTH: COMPARISON OF PENICILLIN LEVELS IN PLASMA OF NORMAL MEN AFTER SINGLE ORAL DOSES

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THE observations reported in this paper were designed primarily to compare the absorption after oral administration of 2 forms of penicillin, one the potassium salt of benzylpenicillin, or penicillin G, and the other benzethacil,* a recently introduced repository form of penicillin.[1] A number of studies were carried out with the following factors kept constant: (a) a single dose of 200,000 units of penicillin was used in every instance; (b) this was given in tablet form; (c) the same subjects were used in all the studies. The effect of the following factors on the levels of penicillin in plasma was studied and compared: (a) administration before or after a meal, (b) incorporation of sodium citrate as a buffer in the tablets of potassium penicillin G, and (c) the type of benzethacil tablet as judged by its disintegration time in artificial gastric juice.

These studies were carried out in an attempt to determine an optimum dosage form for prophylaxis against hemolytic streptococcal infections and hence in the prevention of recrudescences of rheumatic fever. The benzethacil was chosen for investigation because of indications that penicillin may be demonstrated in the plasma after oral administration of this compound for longer periods than after other available oral forms of penicillin.[1-4] Potassium penicillin G was chosen for comparison because it was considered to be reliable, generally available, and economical. Moreover, penicillin levels in plasma after oral administration of potassium penicilin G seemed to compare favorably with those reported after other forms with which they have been directly compared.[5-8]

One report,[9] published since the present studies were completed, compared potassium penicillin G and benzethacil by mouth in the same 6 subjects. Both of these forms in single doses of 300,000 units gave satisfactory levels for four but not for six hours, and the potassium penicillin G appeared to give higher average levels. In that study the potassium penicillin G was given in the form of a tablet free of excipients and binds, while the benzethacil was given in a stable suspension. The doses were all given at 8 A.M., presumably before breakfast, although this is not specifically mentioned.

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*Benzethacil is the generic name for N-N'dibenzylethylenediamine dipenicillin; it was generously supplied under the trade-mark "Bicillin" by Dr. Edward F. Roberts of Wyeth Laboratories who also supplied the preparations of potassium penicillin G.

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MATERIALS AND METHODS

The subjects were normal adult males, 25 to 35 years old, most of them weighing between 170 and 190 pounds (average 180 pounds). In every experiment each subject received a single dose of 200,000 units of penicillin as one of the following preparations: (a) Potassium penicillin G, 100,000 units with sodium citrate (0.57 Gm.) incorporated in each tablet, disintegration time thirty to forty-five minutes. (b) Soluble crystalline potassium penicillin G, unbuffered, 100,000 units per tablet. (c) Benzethacil, 200,000 units per tablet, disintegration time ninety to one hundred and twenty minutes ("hard"). (d) Benzethacil, 100,000 units per tablet, disintegration time thirty to forty-five minutes ("intermediate"). (e) Benzethacil, 200,000 units per tablet, disintegration time ten to fifteen minutes ("soft").

The dose was given either about one-half hour before breakfast or about one-half hour after breakfast. The study was conducted so that each subject acted as his own control for all of the studies. Moreover, one or two variables were compared at the same time so that on any given day 2 or 4 experiments were being carried out simultaneously, each on three to six subjects, and the plasmas from all of these subjects were assayed at the same time, with minor exceptions. The experiments were then repeated, rotating the subjects until all had participated in each experiment. Doses were given on no more than two days in any one week so that a minimum of two days and more often five or more days elapsed between successive experiments in the same subject.

The sequence in which the experiments were conducted was as follows: experiments I to IV were run simultaneously; on the first day 6 subjects received buffered potassium penicillin G, 3 of them before (Experiment I) and 3 after breakfast (Experiment II), while 6 others took the benzethacil "hard" tablet, 3 of them before (Experiment III) and the others after breakfast (Experiment IV). This was repeated on 3 subsequent occasions with rotation of the groups until each of them had participated in all the experiments. At the completion of these 4 experiments, the 6 subjects who on the last day had taken the buffered penicillin G, then took the same preparation after breakfast on each of the following seven days (Experiment VI), while the other 6 all took benzethacil tablets daily after breakfast (Experiment VII). After the seventh daily dose, a series of blood samples were obtained for assay from each subject and he was then switched to the alternate preparation; a second series of assays were again done after the seventh daily post-breakfast dose. Experiment V was essentially a repetition of Experiment IV carried out in 8 of the subjects, randomly chosen; in this experiment, however, each subject took a "standard" meal consisting of fruit juice, milk, buttered toast, and coffee with cream and sugar, whereas the meals in the other experiments varied considerably and were usually more substantial and contained more proteins. Subsequently, the unbuffered potassium penicillin G tablets were studied, 6 of the subjects taking them before (Experiment VIII) and 6 after breakfast (Experiment IX) and the experiment repeated two days later, with the subjects reversing the relation of the dose to the meal. The "intermediate" tablet of benzethacil was then tested on one day, 6 subjects taking it before (Experiment X) and 6 after breakfast (Experiment XI). Finally, the "soft" tablet of benzethacil was given before breakfast to the latter (Experiment XII) and after breakfast to the former (Experiment XIII). The last 4 experiments were not repeated with "cross-overs."

Citrated venous blood samples were obtained immediately before each dose in about half of the experiments; these invariably showed no penicillin. Blood was drawn one, two, four, and six hours after the dose in all experiments and also at eight hours in the experiments involving benzethacil. The plasma was separated promptly and stored in a refrigerator at 5° to 10° C. until all the samples were collected, and the assays were then carried out on the same day. In some instances, part of each sample was stored at -20° C. to permit repetition of the assays later if necessary.

Penicillin assays were carried out with every specimen by the serial twofold dilution method in brain heart infusion broth (Difco, pH 7.4±) using *Streptococcus 98* as the test organism, 0.5 ml. of a 10^{-4} dilution of the fully grown culture being added to an

equal volume of the plasma dilutions. The smallest amount of penicillin detectable in these tests was 0.03 unit per milliliter of plasma. Duplicate assays were carried out on the same sera, either at the same time or later, by a plate-diffusion method using *Sarcina lutea* in agar on which were placed filter paper discs saturated with a constant volume of undiluted or diluted plasma. However, since the results obtained with this method did not show any constant or significant qualitative or quantitative difference from those obtained by the broth-dilution method, only the results of the latter are reported. Moreover, in several experiments, the subjects' own control ("0" hour) plasma was incorporated in the diluting broth for the penicillin assays (including the penicillin control) without significantly affecting the results.

RESULTS

Since the same subjects were used throughout these studies, it seems appropriate to present the actual penicillin levels obtained in all experiments in every subject; these are shown in Table I. Certain broad generalizations appear to be warranted from inspection of these data: (a) There were moderate to marked differences in the concentrations of penicillin in the plasma of different subjects in each experiment, that is, at the same interval after ingestion of the same preparation and in the same relation to the meal. (b) These variations among the subjects within the same experiment were greater in some experiments than in others, or, put another way, there was greater uniformity in the plasma concentrations in different subjects after administration of some preparations as compared with those obtained after other preparations or even after the same preparation given in a different relation to the meal. (c) Marked variations in plasma concentrations occurred in the same subject in different experiments. (d) These variations in penicillin levels in plasma obtained in the same subject in different experiments were by no means uniform or similar in different subjects. (e) At the specified periods after a dose, the number of subjects who failed to show demonstrable levels of penicillin in the plasma varied in different experiments. In addition, in some experiments certain of the subjects did not have assayable penicillin in any of the specimens or had penicillin demonstrable in only one of the specimens that were assayed. This aspect is summarized in Table II for all the experiments. (f) Finally, it must be emphasized that these generalizations and any comparisons of the various preparations must be interpreted rather broadly because of the crude biological assay method employed. Although the significance of the data is considerably enhanced by the employment of the same subjects throughout, nevertheless, only gross or consistent differences can be considered to be meaningful.

Absorption of Buffered Potassium Penicillin G Before and After Breakfast (Experiments I and II).—Penicillin was demonstrable in the plasma of every subject one and two hours after ingestion of the dose of 200,000 units, and assayable levels were present in most of the subjects at four hours, irrespective of whether the dose was taken before or after breakfast. In only one subject in each experiment was penicillin still demonstrated at six hours. The average levels for all 12 subjects were the same at each interval. These findings suggest that this buffered preparation of potassium penicillin G was more or less uniformly absorbed; moreover, the absorption was not significantly affected by the meal.

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TABLE I. CONCENTRATIONS OF PENICILLIN IN PLASMA AFTER SINGLE ORAL DOSE OF 200,000 UNITS

EXPERIMENT*	HOUR AFTER DOSE†	SUBJECT A	B	C	D	E	F	G	H	J	K	L	M	MEAN
		Units per milliliter‡												
I Buffered potassium penicillin G before breakfast	1	0.5	1.0	0.5	0.5	0.5	0.25	0.5	0.25	0.25	0.13	0.25	0.25	0.40
	2	0.25	0.13	0.13	0.13	0.25	0.25	0.13	0.06	0.13	0.06	0.13	0.13	0.15
	4	0.06	0.03	0.03	0	0.03	0.13	0	0	0.03	0	0	0.06	0.03
	6	0	0.03	0	0	0	0	0	0	0	0	0	0	0
II Same as I after breakfast	1	0.13	0.25	0.5	0.25	0.25	0.06	0.25	1.0	0.5	0.5	0.5	0.13	0.36
	2	0.25	0.13	0.13	- - -	0.5	0.13	0.13	0.25	0.25	0.13	0.25	0.13	0.19
	4	0.06	0	0.03	0.03	0.03	0	0.06	0.13	0.25	0.03	0.03	0.06	0.06
	6	0	0	0	0	0	0	0	0	0.03	0	0	0	0
III Bicillin, "hard" tablet before breakfast	1	0	0.03	0	0.03	0.03	0.03	0	0	0.03	0.06	- - -	0.03	0.023
	2	0.03	0	0	0.03	0.03	0	0	0	0	0.03	0.03	0	0.013
	4	0	0	0	0.03	0	0	0	0.13	0	0	0.03	0	0.015
	6	0	0	0	0	0	0.06	0.06	0.03	0	0	0	0	0.013
	8	0	0	0	0	0	0	0.03	0	0	0	0	0	0
IV Same as III after breakfast	1	0.03	0	0	0.03	0	0	0	0	0	0	0	0	0.005
	2	- - -	0	0	0.06	0.03	0	0	0	0	0	0	0.06	0.013
	4	- - -	0.06	0.13	0.06	0	0.03	0	0.25	0.06	0.25	0.13	0.25	0.11
	6	- - -	0	0	0.03	0.03	0.03	0	0.06	0	0.06	0.06	0.06	0.03
	8	- - -	0	0	0	0	0	0	0.03	0	0.03	0.03	0.03	0.01
V Same as IV after "standard" breakfast	1	- - -	0.06	0	- - -	0	0	- - -	0	0	0	- - -	0	0.008
	2	- - -	0.06	0	- - -	0	0	- - -	0.06	0	0.06	- - -	0.03	0.026
	4	- - -	0	0.06	- - -	0.03	0.03	- - -	0.06	0.03	0.03	- - -	0.06	0.038
	6	- - -	0	0	- - -	0	0.03	- - -	0	0	0.03	- - -	0	0.008
	8	- - -	0	0	- - -	0	0.03	- - -	0	0	0	- - -	0	0
VI Same as II seventh daily dose	1	0.25	0.5	0.13	0.5	0.13	0.25	- - -	0.25	0.25	0.25	1.0	1.0	0.41
	2	0.5	0.13	0.06	0.25	0.25	0.13	- - -	0.5	0.13	0.13	0.13	0.5	0.25
	4	0.06	0.03	0.03	0.06	0.03	0	0.06	0.03	0	0.03	- - -	0.06	0.035
	6	0	0	0	0.03	0	0	0.03	- - -	0	0	0	0	0.005
VII Same as IV seventh daily dose	1	0	0	0.03	0.06	0.03	0	0	0	0	0	0	0	0.01
	2	0.03	0.03	0.13	0.13	0.03	0	0	0.06	0	0.06	0	0.03	0.04
	4	0.03	- - -	0	- - -	0	0	0.06	0.06	0.06	0.03	0	0	0.024
	6	0	0	0.06	- - -	0	0.03	0.06	0	0	0.03	0	0	0.016
	8	0	0.03	0	- - -	0	0	0	0	0	0	- - -	0	0
VIII Same as I unbuffered	1	0.06	0.13	0	- - -	0.25	0.06	0.13	0.25	1.0	0.5	0.5	0.5	0.31
	2	0	0.06	0	0.06	0.06	0.03	0.03	0.06	0.25	0.13	0.06	0.13	0.07
	4	0	0	0	- - -	0	0	- - -	0	0.03	0.03	- - -	0.03	0.01
IX Same as II unbuffered	1	0.25	0.5	0.25	0.5	0.13	0.06	0.25	- - -	0.13	0.03	- - -	0.06	0.21
	2	0.06	0.06	0.13	0.06	0.06	0.03	0.13	0	0.06	0	- - -	0.03	0.05
	4	0	0	0	0.03	0	0	0	0	0	0	- - -	0	0

X	Bicillin "Intermediate" before breakfast	1							0.06	0.13	0.06	0.03	0.03	0.13	0.07
		2							0.06	0.13	0.06	0.03	0.03	0.03	0.06
		4							0.03	0	0	0	0	0	0.005
XI	Same as X after breakfast	1	0.03	0.13	0.25	0.25	0.25	0.03							0.16
		2	0.13	0.03	0.13	0.13	0.25	0.06							0.13
		4	0.06	0	0.03	0.06	0.03	0							0.03
XII	Bicillin "soft" tablet before breakfast	1	- --	0.06	0.03	0.13	0.03	0.03							0.05
		2	0.03	0.03	0.06	0.13	0.06	0.03							0.06
		4	0	0	0.03	0.03	0	0							0.01
		6	0	0	0	0.03	0	0							0.005
XIII	Same as XII after breakfast	1							0	0.06	0	0.03	0.13	0	0.04
		2							0	0.13	0	0.03	0.06	0	0.04
		4							0.03	0.03	0.03	0.03	0.03	0.06	0.04
		6							0.03	0	0.03	0	0	0.03	0.015
Weight (pounds)			184	210	215	185	186	188	142	170	162	174	175	173	180

*Disintegration time in artificial gastric juice: "Hard" tablet = 90-125 minutes; "Intermediate" = 30-45 minutes; "Soft" = 10-15 minutes. The tablets of potassium penicillin G, buffered and unbuffered, as used in Experiments I, II, VI, VIII, and IX and the "Intermediate" tablet of benzethacil in Experiments X and XI each contained 100,000 units; in the remaining experiments the 200,000 units were contained in a single tablet.

†Assays were done before the dose in Experiments I to VII, inclusive; they were done at one, two, four, and six hours in all experiments, and also at eight hours in the experiments with Bicillin. For brevity the data are not shown for periods in which none of the subjects showed any demonstrable levels.

‡Twofold tube dilution method using *Streptococcus 98;* lowest value assayed = 0.03 unit per milliliter; 0 = <0.03 unit per milliliter for individual specimens, and <0.005 unit per milliliter for mean values. — = not done.

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TABLE II. FAILURE TO DEMONSTRATE DETECTABLE LEVELS OF PENICILLIN AT VARIOUS INTERVALS AFTER SINGLE ORAL DOSE OF 200,000 UNITS

EXPERIMENT NO.*	NO. OF SUBJECTS TESTED	NUMBER WITH LESS THAN DETECTABLE LEVEL (< 0.03 UNIT/ML.)†							
		HOURS AFTER DOSE						IN ALL SPECIMENS	IN ALL BUT ONE SPECIMEN
		0	1	2	4	6	8		
I	12	12	0	0	5	11	--	0	0
II	12	11[1]	0	0[1]	2	11	--	0	0
III	12	12	4[1]	7	9	9	11	1	4
IV	12	12	10	8[1]	2[1]	4[1]	7[1]	1	3
V	8	8	7	4	1	6	7	0	3
VI	12	12	0[1]	0[1]	2[1]	9[1]	--	0	0
VII	12	12	9	4	5[2]	7[1]	9[2]	1	3
VIII	12	--	1[1]	2	6[2]	12	--	1	1
IX	11	--	0[1]	2	10	11	--	1	1
X	6	--	0	0	5	6	6	0	0
XI	6	--	0	0	2	6	6	0	0
XII	6	--	0[1]	0	4	5	6	0	0
XIII	6	--	3	3	0	4	6	0	0

*See Table I.

†Occasional specimens were lost or not obtained in some of the subjects, as noted in Table I; the number not done is indicated by superscripts; — indicates that specimens were not taken from any subjects at this hour.

However, there is a slight suggestion from the data that the rate of absorption may have been more variable, and that in some subjects absorption was delayed when the dose was taken after the meal. Thus, the penicillin level was higher at two hours than at one hour in 3 subjects when they took the dose after a meal, but in no instance was that true when the dose was taken before breakfast. Moreover, only 2 of the subjects failed to show assayable levels at four hours when they took the dose after breakfast, whereas 5 failed to yield such levels at the same intervals when they took the tablets before the meal.

Absorption of Benzethacil ("Hard" Tablet) Before and After Breakfast (Experiments III, IV, and V).—The obvious and outstanding feature of these 3 experiments, as seen from Tables I and II, is the irregularity with which penicillin was demonstrable in the plasma of the different subjects, at each of the intervals and in each of the 3 experiments. One subject in Experiment III and another in Experiment IV failed to show an assayable level at any of the intervals studied, and 3 or 4 other subjects in each of the 3 experiments had demonstrable penicillin in only one of the specimens of plasma. As far as it is possible to judge from such irregular findings, absorption of the dose taken after breakfast appeared to be somewhat delayed, so that penicillin was demonstrable after the postcibal dose less often in the samples of plasma taken at one and two hours, but more often at four and six hours. The average levels were somewhat higher at one hour after the fasting dose and at four and six hours when the dose was taken after breakfast.

Effect of Giving Buffered Potassium Penicillin G or Benzethacil Daily After Breakfast (Experiments VI and VII).—When the 200,000 unit dose of penicillin was given after breakfast on seven consecutive days, either as the buffered potassium salt of penicillin G or as the hard tablet of benzethacil, the

levels of penicillin obtained in the plasma after the seventh daily dose corresponded, in general, with those previously obtained from the first dose of the same form of penicillin given after breakfast. Moreover, the levels were obtained after the benzethacil tablets with similar irregularity on both occasions. Likewise, the mean levels obtained at the different intervals were very similar in Experiment VI to those found in Experiment II, and the average levels in Experiment VII were not much different from those observed in Experiments IV and V.

Absorption of Unbuffered Potassium Penicillin G Before and After Breakfast (Experiments VIII and IX).—In previous studies in this and other laboratories[10-13] it was found that absorption of oral penicillin was irregular and unreliable if given directly after a meal. In some of those studies unbuffered penicillin was used which, in the earlier of the studies, was probably a relatively impure mixture of various penicillins. The effect of sodium citrate on the absorption of penicillin, as reported by others, varied particularly in relation to the ingestion of food[14-17] and the amount of citrate included. Because no significant difference was noted between the blood levels obtained when the buffered potassium penicillin G was given before or after breakfast (Experiments I and II), it was of interest to investigate the effect of a meal on the absorption of unbuffered potassium penicillin G and at the same time compare the levels with those obtained with the buffered preparation which contained about 1.15 Gm. of sodium citrate per dose.

In Experiments VIII and IX, as can be seen from Tables I and II, the unbuffered tablets of potassium penicillin G yielded levels which were more irregular and generally, though not uniformly, lower than those previously obtained with the buffered tablets in Experiments I, II, and VI; this was true regardless of whether the unbuffered tablets were taken before or after breakfast. Moreover, there was no suggestion in any of the subjects of delay in absorption of the unbuffered tablets when they were given after the meal. In these experiments, although some subjects had higher levels after the postcibal unbuffered dose, more of them achieved higher levels from the fasting dose and more of them had assayable levels at four hours in Experiment IX. The mean plasma levels were lower at each interval after the unbuffered doses as compared with the buffered doses; also, at the first hour after ingestion of the unbuffered tablets, the mean value was higher from the fasting dose than from the dose taken after breakfast.

Effect of Disintegration Time of the Tablet on Absorption of Benzethacil Before and After Breakfast (Experiments X-XIII).—Boger and Beatty[11] compared the penicillin levels and urinary excretion after ingestion of 2 types of tablets of procaine penicillin having different disintegration times; the levels obtained with the tablet that disintegrated slowly were lower, more irregular, slower to rise, and much less penicillin was recovered in the urine. Because the tablets of benzethacil used in Experiments III, IV, V, and VII had a long disintegration time in artificial gastric juice, additional observations were made with 2 tablet forms that disintegrated more rapidly, one in thirty to forty-five

minutes, and the other in ten to fifteen minutes. With both of these tablets absorption was more rapid and more regular and the average levels of penicillin were higher than after ingestion of the hard tablet used in the previous experiments. This was true with the tablets taken either before or after breakfast. Although direct comparisons with respect to the effect of the meal were not made in the same subjects for each of these 2 tablets, the results suggest that absorption was more complete and higher levels were obtained when the "intermediate" tablet was taken after breakfast, while with the "soft" tablet the effect of the meal was to delay absorption in some subjects.

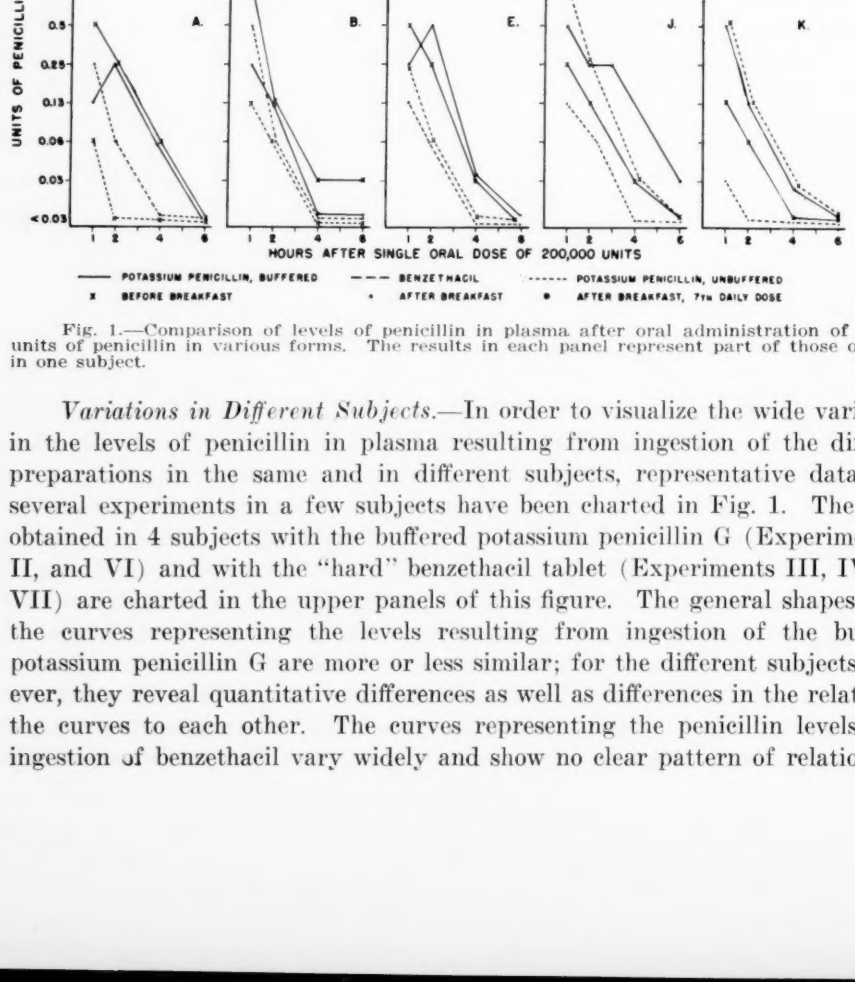

Fig. 1.—Comparison of levels of penicillin in plasma after oral administration of 200,000 units of penicillin in various forms. The results in each panel represent part of those obtained in one subject.

Variations in Different Subjects.—In order to visualize the wide variations in the levels of penicillin in plasma resulting from ingestion of the different preparations in the same and in different subjects, representative data from several experiments in a few subjects have been charted in Fig. 1. The levels obtained in 4 subjects with the buffered potassium penicillin G (Experiments I, II, and VI) and with the "hard" benzethacil tablet (Experiments III, IV, and VII) are charted in the upper panels of this figure. The general shapes of all the curves representing the levels resulting from ingestion of the buffered potassium penicillin G are more or less similar; for the different subjects, however, they reveal quantitative differences as well as differences in the relation of the curves to each other. The curves representing the penicillin levels after ingestion of benzethacil vary widely and show no clear pattern of relationship.

In the lower part of the figure are charted the penicillin levels obtained in 5 other subjects after single doses of the buffered and unbuffered potassium penicillin G taken before and after breakfast. These curves are all generally similar in character; those representing the levels resulting from the buffered preparation show no uniform effect of the meal, whereas lower levels resulted more often from the unbuffered tablets when they were taken after breakfast as compared to those following the fasting dose of the same tablets.

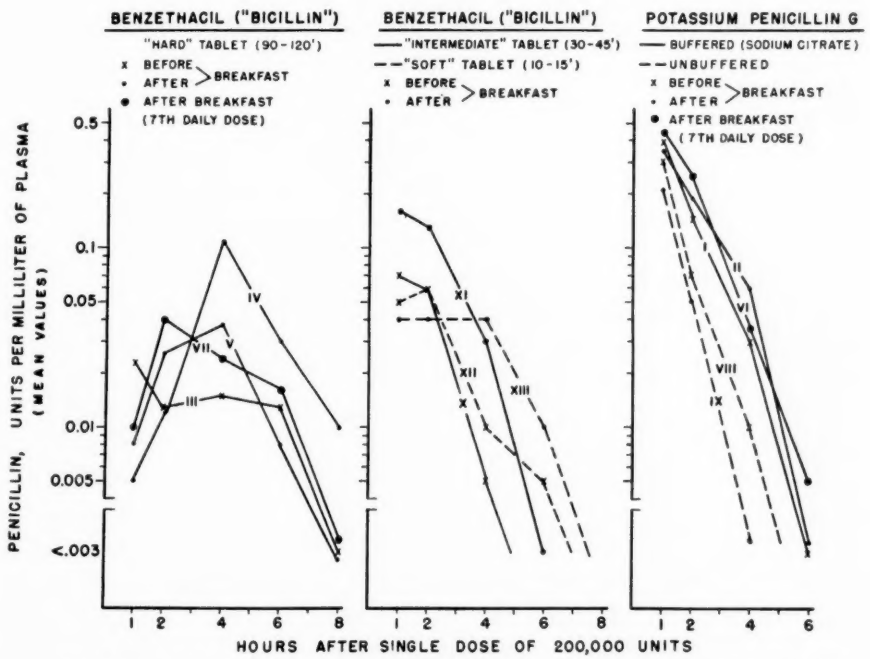

Fig. 2.—Mean level of penicillin in plasma following oral administration of 200,000 units of penicillin in different forms, before and after breakfast. The Roman numeral indicates the number of the experiment as designated in Table I.

Comparison of Average Levels Obtained With Different Preparations.—In order to visualize the over-all results, the mean values obtained in each of the experiments have been charted; the resulting curves fall into 3 patterns, shown in the separate panels in Fig. 2. The hard benzethacil tablet (left panel) yielded curves with late rises reaching peaks at two to four hours and higher peaks when the doses were taken after breakfast. The mean levels obtained with potassium penicillin G (right panel) gave the most uniform curves; these showed early peaks and steep declines. These curves reveal little or no difference in the levels obtained with the buffered penicillin when given before or after breakfast. They also reflect the poorer absorption (and hence lower average plasma levels) of the unbuffered as compared with the buffered potassium penicillin tablet and the better and more sustained plasma levels following ingestion of the unbuffered tablet when taken fasting as compared with those obtained when the dose was taken after breakfast. The "intermediate" and "soft" tablets (center

panel) gave curves for the mean levels which were intermediate in character, suggesting slow or prolonged absorption, with the mean levels declining only after two or four hours. These curves also reflect the higher and more sustained levels from the "intermediate" tablet taken after breakfast and the delayed absorption of the softer tablet when taken after a meal.

DISCUSSION

In the present studies, no significant difference was demonstrated between the levels of penicillin obtained in the plasma when buffered potassium penicillin G was given before or after breakfast. This was somewhat unexpected in view of many earlier studies which indicated that absorption of penicillin was irregular and often markedly reduced when an oral dose was given with, or soon after a meal. Two possible explanations were considered. (a) In many of the earlier studies the preparations of penicillin that were used were relatively impure; they consisted of mixtures of various penicillins and usually unbuffered and amorphous preparation of their sodium or calcium salts. That some of the known forms of penicillin (for example, penicillins F, G, X, and K) are absorbed or excreted at different rates[18, 19] is now well recognized, although in one study such differences were not noted in the case of oral administration of 4 forms of penicillin.[13] Also, some of the earlier preparations, especially those of the relatively impure and amorphous sodium penicillins, were much less stable than the highly purified crystalline potassium salt of penicillin G that is now generally used. (b) The buffer of sodium citrate may also have contributed to the better absorption after a meal. Reference was made earlier to the divergent reports on the effects of citrate on absorption of oral penicillin in relation to food. The effect of buffers, however, may be influenced by the manner in which they are given. Barach and his co-workers[20] noted that aluminum hydroxide gel given one-half hour before an oral dose failed to influence the resulting levels of penicillin; however, when the gel was mixed with the same dose of penicillin before ingestion, higher levels were obtained quite regularly as compared with the same amount of penicillin alone. In those experiments the penicillin was given between meals. In the present study, the incorporation of the citrate in the tablet may have given greater protection to the penicillin against the action of the gastric contents.

It did not seem feasible or desirable to study, in a carefully controlled manner, all of the possibilities and variables involved. However, from the practical point of view, it did appear worth while to compare the buffered and unbuffered preparations of potassium penicillin G. The results confirmed some of the earlier reports; the unbuffered preparation yielded generally lower levels which were still further reduced when the doses were taken after breakfast. Other buffers were not tested but some, like calcium carbonate, have been reported as highly effective.[21, 22] Moreover, buffers may not be necessary or useful in infants, while they are in children.[22, 23]

Benzethacil, in the tablet which was originally tested and which had the long disintegration time, yielded the most irregular and unpredictable levels

in the blood. In general, absorption of penicillin from this form appeared to be delayed, and higher peak levels were attained when these tablets were given after breakfast as compared with fasting doses, but the results were still irregular. That this delayed and irregular absorption was related, at least in part, to the long disintegration time of the tablet was suggested by the more regular absorption of the "intermediate" tablet, which likewise yielded generally higher and better sustained levels in the plasma, when taken after breakfast. The "soft" tablet, however, yielded erratic results when given after a meal, 3 of 6 subjects having no demonstrable levels of penicillin in their plasma at one and two hours.

The possibility of obtaining higher and more sustained blood levels with drugs which inhibit the renal excretion of penicillin was not investigated. Boger and his co-workers, however, have shown quite clearly that this may be accomplished with probenecid (Benemid). When a single dose of 1 Gm. of probenecid is given (incorporated in the same tablets) with 400,000 units of potassium penicillin G,[23, 24] or when the probenecid is given along with benzethacil, either 1 Gm. of the former and 300,000 units of the latter or twice these amounts,[9] the resulting peak levels of penicillin in the plasma are about the same and are both reached rapidly, but the levels thereafter are significantly higher and more sustained as compared with those obtained in the same subjects with the same dose of penicillin but without the probenecid.

Inasmuch as this study was motivated by the need for choosing a proper antibacterial agent for prophylaxis against recurrences of rheumatic fever, a brief comment on this aspect is warranted. The properties desired in such an agent are: (a) proved effectiveness in preventing hemolytic streptococcal infections, (b) simplicity of administration and acceptability to the patient, (c) reliable and reproducible absorption, (d) low toxicity, (e) economy, (f) if given by mouth, absorption should preferably be uninfluenced by food. Which of any number of available preparations might best satisfy these criteria must, in the end, depend on observations in a large series of randomized cases carefully followed and controlled, both clinically and bacteriologically. From the present data it can be said only that the buffered tablets of potassium penicillin G should be preferable to any of the other oral preparations that were tested. Moreover, from the available reports, such a preparation would seem to be as good as any others with which it has been compared, at least as regards its absorption, with the possible exception of the combination of potassium penicillin G with probenecid. The latter combination, however, has the disadvantage inherent in the prolonged use of 2 potent and potentially sensitizing agents. Moreover, the effects of prolonged use of probenecid in people without gout have not yet been adequately studied.

SUMMARY AND CONCLUSIONS

The levels of penicillin in the blood (plasma) have been determined in the same 12 subjects after administration of single doses of 200,000 units of penicillin by mouth, before and after breakfast, and in several forms, namely, as

tablets of buffered and unbuffered potassium penicillin G and as benzethacil ("Bicillin") in 3 types of tablets having different disintegration times.

The most uniformly absorbed among these preparations was the tablet of potassium penicillin G buffered with sodium citrate; this yielded the highest peak levels, and its absorption was not significantly affected by the meal. The unbuffered tablet of potassium penicillin G yielded lower average levels of penicillin in the plasma, and these were further reduced and were somewhat irregular when the dose was given after breakfast.

Benzethacil in the "hard" tablet, which was slow to disintegrate, was absorbed very erratically; with this preparation penicillin was generally slow to appear in the plasma, and the levels rose irregularly, but higher peaks were reached in some subjects when the dose was given after breakfast. Tablets of benzethacil which disintegrated faster yielded higher peak concentrations of penicillin and these were achieved more rapidly and were better sustained if given after the meal. The levels obtained with the latter preparations were intermediate between those resulting from potassium penicillin G and those following ingestion of the hard tablet of benzethacil.

There was no cumulative effect on the levels of penicillin in the plasma following 7 daily doses of either the buffered potassium penicillin G or the benzethacil, each given after breakfast.

Of the preparations tested, therefore, the most reliable and most desirable from the point of view of absorption is the buffered tablet of potassium penicillin G.

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COMPARISON OF THE IN VIVO SODIUM-REMOVING ACTIVITY OF VARIOUS TYPES OF ION EXCHANGE RESINS IN RATS

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ION exchange resins have now come into use for the control of sodium absorption in various types of edema, and are being administered experimentally in some cases of hypertension. In fact, resins are proving effective in any situation where restriction of gastrointestinal absorption of sodium is of importance (Arnold,[1] Dock,[2] Hay and Wood,[3] Danowski and associates,[4] Kahn and Emerson,[5] Martz, Kohlstaedt and Helmer[6, 7]). The resins in use at the present time have several disadvantages as therapeutic measures, i.e., large daily doses of an insoluble material; acidosis which, although generally compensated, may overload the kidneys; loss of sodium absorbing capacity of the resin due to uptake of potassium and other cations. A large variety of resins of various types have become available during the past two or three years, and the present paper is concerned with a study of the in vivo activity in rats of samples representative of various types of resins. Carboxylic acid resins, sulfonic acid resins, phosphonic acid resins, liquid resins, anion exchange resins, and various combinations of these types have been investigated.

METHODS

Male rats of the Wistar strain were used for all experiments. The animals were placed in metabolism cages, two rats to a cage, and given food and distilled water ad libitum. A standard ground rat diet was fed to all animals. Resin-containing diets were prepared by mixing the desired amounts of resin in this standard diet. Table I gives the composition of all the diets reported in this paper. These will be referred to in the text either by number or by the specific resin which is under consideration in that section. Food and water consumption and body weight were measured daily. Urine and feces were collected from each cage daily during test periods and pooled for three-day samples. The animals were maintained on either the standard or test diet for seven days, then urine and feces samples were collected for two successive three-day periods. Each resin test was preceded and followed by a test on the standard diet. The intake of food and resin was not controlled, but in general the rats compensated for the noncaloric resin by eating larger amounts of the resin-containing diets.

Feces, urine, and food samples were analyzed for chloride, phosphate, sodium, and potassium. The use of distilled water in the drinking bottles precluded the necessity of analyzing the water except when the two liquid resins were tested. Urine chlorides were determined by the modified Volhard-Harvey titration method, and urine inorganic phosphate by the Fiske and Subbarow method. Urinary sodium and potassium were determined on suitably diluted samples using the Beckmann flame photometer. Feces samples were dried for twelve to eighteen hours in an oven at 100° C. They were then ground and thoroughly mixed. Chlorides were determined on 2-gram samples of the dried feces by the open Carius method. For the determination of fecal phosphate, sodium, and potassium, a 500 mg. sample

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TABLE I. COMPOSITION AND *in vivo* SODIUM BINDING CAPACITY OF RESIN-CONTAINING DIETS

DIET NO.	CATION EXCHANGER		ANION EXCHANGER	TYPE OF CATION EXCHANGER	Na BOUND BY RESIN (MEQ./GM.)
	H CYCLE 5.9%	K CYCLE 2.9%	1.2%		
1	Amberlite XE-64*	Amberlite XE-88	Amberlite XE-58	Carboxylic acid	0.39
2	Amberlite XE-64	Amberlite XE-88		Carboxylic acid	0.38
3	Amberlite XE-112	Amberlite XE-88	Amberlite XE-58	Carboxylic acid	0.88
5	Duolite DS-517†	Duolite DS-517	Amberlite XE-58	Sulfonic acid	0.23
6	Dowex 50W†	Dowex 50W	Amberlite XE-58	Sulfonic acid	0.44
7	Amberlite XE-103	Amberlite XE-103		Carboxylic acid	0.83
9	Duolite DS-566‖	Duolite DS-565		Carboxylic acid	0.09
10	Amberlite XE-122‖	¶		Carboxylic acid	0.05
15	Dowex 50W‡	Dowex 50W		Sulfonic acid	0.17
16	Amberlite XE-64§			Carboxylic acid	0.33
17	Amberlite XE-69§			Sulfonic acid	0.12
18	Amberlite XE-64	Amberlite XE-88	Duolite A-41	Carboxylic acid	0.33
20	Duolite C-60§			Phosphonic acid	0.27
21	Amberlite XE-64	Amberlite XE-88	Amberlite XE-114	Carboxylic acid	0.45
24	Nalco X-219	Nalco X-219 (K)	Amberlite XE-58	Phosphonic acid	0.09

*"Carbo-Resin" (Carbacrylamine Resins, Lilly).
†NH_4 cycle.
‡Glycine cycle.
§7.15%; K bicarbonate 1.19%.
‖Liquid resins, concentration given is per cent in drinking water.
¶0.59% potassium acetate.

of the dried, ground feces was digested by a wet-ashing method using a 1:1 mixture of concentrated sulfuric and nitric acids. After digestion was complete the volume was made up to 50 ml. with distilled water, and the determinations made on aliquots of this solution. Phosphate was determined by the Fiske and Subbarrow method. Sodium and potassium were determined on 1:5 dilutions of the digested and diluted feces samples employing standards containing sulfuric acid in a concentration approximating that of the sample. Food samples were analyzed by the methods used for the feces.

RESULTS

Carboxylic Acid Resins.—Two carboxylic acid cation exchange resins were compared with our standard, Amberlite XE-64, for their ability to increase the fecal excretion of sodium. Both of these resins proved to be more effective than Amberlite XE-64 for binding sodium in the gastrointestinal tract. Table II, part 1, shows the urinary and fecal excretion of sodium, potassium, phosphate, and chloride during feeding of these resins and includes, for comparison, similar experiments with Amberlite XE-64 (diet 1 and diet 2).

The figures in Tables II and III for the amount of sodium, potassium, chloride, and phosphate excreted are based on the dietary intake of the rats. Each figure represents the milliequivalents (meq.) of that ion excreted for every 100 Gm. of diet consumed by two rats in a three-day period.

In the test with Amberlite XE-112 (diet 3) some resin in the potassium cycle was included in the diet as well as an anion exchanger. Amberlite XE-103 (diet 7) is a colloidal form of resin. There was no anion exchanger of comparable physical properties available, so the results with this cation exchanger should be compared with diet 2.

Amberlite XE-112 bound twice as much sodium per gram of resin as did Amberlite XE-64, and the colloidal form of resin, Amberlite XE-103, was

TABLE II. SODIUM, POTASSIUM, CHLORIDE, AND PHOSPHATE EXCRETION BY RATS FED ION EXCHANGE RESINS

DIET	URINE					FECES					Na BOUND BY RESIN
	Na	K	cl	PO_4	% INGESTED Na EXCRETED IN URINE	Na	K	cl	PO_4	% INGESTED Na EXCRETED IN FECES	MEQ./GM.
	MEQ./100 GM. DIET					MEQ./100 GM. DIET					
					Part I						
Control	6.12	8.08	5.03	5.42	42.3	1.73	1.54	1.77	55.4	11.9	0.39
1	3.04	9.35	4.37	13.78	21.6	5.17	3.88	1.54	33.8	36.8	
Control	7.27	7.94	6.56	5.82	50.3	1.36	1.35	1.61	51.2	9.4	0.88
3	0.87	9.87	5.28	20.40	6.3	9.05	5.98	1.47	28.8	65.1	
Control	10.72	7.57	11.29	2.99	58.7	2.94	2.44	2.60	85.2	16.1	0.38
2	6.43	13.30	8.02	10.30	35.3	6.31	4.76	2.99	58.5	39.1	
Control	5.33	10.20	7.65	1.90	29.2	3.19	2.96	3.07	79.3	17.5	0.83
7	2.64	10.52	6.30	17.30	13.6	10.50	8.44	2.92	43.5	54.0	
					Part II						
Control	6.12	4.76	6.04	2.13	31.1	2.52	2.44	2.43	77.8	12.9	0.23
5	5.65	8.52	5.88	6.16	31.8	4.58	3.46	3.43	56.8	25.8	
Control	8.25	6.12	8.27	2.03	41.9	2.03	2.21	2.38	77.7	11.3	0.44
6	4.98	10.55	6.46	5.20	28.7	5.86	3.85	2.46	71.9	33.9	
Control	10.17	9.01	9.78	4.07	50.9	2.34	2.77	2.74	70.2	11.7	0.33
16	5.52	11.40	7.92	14.60	29.2	4.70	4.32	1.69	50.5	24.8	
Control	8.97	8.27	9.92	3.22	44.9	2.26	2.55	2.86	78.5	11.3	0.12
17	7.03	11.60	8.15	8.78	38.1	3.50	4.16	1.50	60.5	19.0	
					Part III						
Control	5.77	4.81	6.38	3.14	24.1	2.18	2.56	2.89	44.7	9.1	0.27
20	6.02	14.22	7.28	11.90	36.7	4.11	3.98	2.80	54.2	25.1	
Control	7.27	7.56	7.56	3.22	37.3	2.41	3.25	2.52	61.0	12.4	0.09
24	6.43	17.70	7.69	17.90	43.8	3.19	4.17	1.98	68.3	21.8	
					Part IV						
Control	5.10	5.44	5.23	2.22	26.6	1.97	2.28	2.46	57.8	10.3	0.09
9	4.50	10.45	5.56	6.22	23.5	3.67	3.69	2.38	59.3	19.2	
Control	3.82	4.52	4.31	2.03	19.9	2.46	2.88	2.98	73.6	12.8	0.05
10	4.50	8.18	4.68	5.48	24.0	2.98	3.31	3.06	61.8	15.9	

slightly more effective, binding two and one-third times as much sodium per gram of resin. In addition to the increase in fecal and decrease in urinary sodium excretion, there was a marked rise in both fecal and urinary potassium excretion due to the greater amount of potassium that was fed the rats. During the control period the diet contained 0.17 meq. of potassium per 100 Gm. of food, whereas the potassium added by feeding a part of the resin in the potassium cycle raised the consumption to 0.32 meq. of potassium per 100 Gm. of food. There was little, if any, change in the excretion of chloride during ingestion of the resin, but the phosphate changes were marked. During resin feeding the fecal phosphate fell and the urinary phosphate excretion rose.

Sulfonic Acid Resins.—The data which have been published concerning the comparative effectiveness of the sulfonic acid and carboxylic acid resins (McChesney,[8] Arnold,[1] McChesney and McAuliff[9]) do not indicate any great difference in the ability of these two types of resins to increase fecal sodium excretion. Greater efficiency in a sulfonic acid type resin with no increase in side effects would be of considerable interest to both clinician and patient, since these resins are less difficult and less expensive than the carboxylic acid resins to produce. Tests were performed with three sulfonic acid resins obtained from three different sources, and two forms of Dowex 50W, one in the ammonia cycle and the second resin prepared in the glycine cycle. Table II, part II, gives the results obtained in rats with these four resins. When these results are compared with Amberlite XE-64 (Table II, part I) it can be seen that in no case did a sulfonic acid resin bind more sodium per gram of resin than did XE-64. Dowex 50W and Amberlite XE-69 appear to have a capacity of about the same magnitude as the standard, XE-64, but none of the sulfonic acid resins tested had the ability to bind sodium in vivo as effectively as did the carboxylic acid resins XE-112 and XE-103.

Other Cation Exchange Resins.—Two samples of phosphonic acid resins and two samples of liquid carboxylic acid resins were tested in rats. Phosphonic acid resins are believed to have a better Na/K ratio in their binding of cations than have the carboxylic acid and sulfonic acid resins. This has been found to be the case in in vitro experiments (Kennedy[10]). However, in rats, as can be seen in Table II, part III, both phosphonic acid resins tested were very much less effective than Amberlite XE-64 in binding sodium in the gastrointestinal tract.

In the clinical use of ion exchange resins, the grittiness of the powder which must be consumed is unpleasant. An investigation was therefore made of some of the liquid cation exchange resins in the hope of finding one which would be readily miscible with water. The results of tests with two liquid resins are presented in Table II, part IV. The resin was mixed with the drinking water in the concentrations indicated in Table I. When the capacity of these resins was calculated, as in Table II, on the basis of milliequivalents of sodium bound per milliliter of resin, the capacity was extremely low. However, when this same calculation was based on the solid content of the resin, the capacity was about the same as that of Amberlite XE-64. Apparently the liquid resins bind

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TABLE III. THE EFFECT OF DIETARY SODIUM CONCENTRATIONS UPON THE EXCRETION OF SODIUM, POTASSIUM, CHLORIDE, AND PHOSPHATE IN RATS FED A CARBOXYLIC ACID CATION EXCHANGE RESIN

DIET	URINE					FECES					
	Na	K	cl	PO_4	% INGESTED Na EXCRETED IN URINE	Na	K	cl	PO_4	% INGESTED Na EXCRETED IN FECES	Na BOUND BY RESIN MEQ./GM.
	MEQ./100 GM. DIET					MEQ./100 GM. DIET					
					Sodium Deficient Diet (0.0039 meq. Na/Gm.)						
Control	8.78	14.12	12.60	15.70	41.1	0.09	0.24	0.43	22.0	23.1	0.04
1	0.10	3.33	4.75	13.50	16.1	0.42	5.90	0.72	1.5	71.2	
					Medium Sodium Diet (0.1402 meq. Na/Gm.)						
Control	6.12	8.08	5.03	5.42	42.3	1.73	1.54	1.77	55.4	11.9	0.39
1	3.04	9.35	4.37	13.78	21.6	5.17	3.88	1.54	33.8	36.8	
					High Sodium Diet (0.1875 meq. Na/Gm.)						
Control	5.57	5.47	5.88	2.16	29.1	2.48	2.95	2.73	71.8	12.9	0.53
1	4.68	9.92	7.10	13.15	25.0	7.14	5.55	3.05	53.7	38.1	
					Very High Sodium Diet (1.118 meq. Na/Gm.)						
Control	84.5	10.4	98.2	15.7	71.3	1.32	0.06	1.21	—	1.2	1.43
1	49.2	14.0	68.2	17.4	41.7	13.90	0.58	1.86	—	12.0	

cations in the rat gastrointestinal tract in a manner similar to the solid resins. There was no evidence of toxicity in this short-term experiment. The liquid resins remain of theoretical interest only, since the great bulk of liquid which would have to be ingested in order to remove any appreciable amount of sodium makes them completely impractical for clinical use.

Anion Exchange Resins.—It has been found that, in man, the addition of an anion resin to the cation combination increases the sodium binding capacity of the cation resin (Martz, Kohlstaedt, and Helmer[7]). In rat experiments McChesney and McAuliff[9] found a slight improvement in the sodium binding capacity of the cation resin when anion exchange resin was added to the mixture fed. However, the fact that the amount of cation resin in the diet was not kept constant makes it difficult to compare the results they obtained in the several test periods. In the experiments reported here the addition of the anion exchange resin Amberlite XE-58 to the cation resins Amberlite XE-64 and Amberlite XE-88 did not appreciably increase the sodium binding capacity of the cation resins. The sodium binding capacity of diet 1 (containing anion exchange resin) was found to be 0.38 meq. per gram, whereas that of diet 2 (no anion resin) was also 0.38 meq. per gram. In a repetition of this experiment diet 1 bound 0.44 meq. per gram and diet 2 bound 0.39 meq. per gram. Apparently the intestinal milieu of the rat is not greatly influenced by the presence of an anion exchange resin in the quantity used in these diets.

In an attempt to find some means of increasing fecal and decreasing urinary chloride excretion, two anion exchange resins were tested. Neither Amberlite XE-114 nor Duolite A-41 increased the fecal excretion of chloride above that found when Amberlite XE-58 was employed.

The Effect of Dietary Sodium Concentration.—The ability of the resins in diet 1 to bind sodium was affected by the composition of the diets being fed to the rats. This became apparent when a new batch of standard rat food was used to prepare the diets. The "high" sodium diet listed in Table III does not contain a great deal more sodium than the "medium" sodium diet, but that little was enough to increase the amount of sodium bound by the resin. The low sodium diet was deficient in sodium, whereas the very high sodium diet contained a great excess of sodium. This very high sodium diet was a synthetic diet used by Meneely, Tucker, and Darby[11] for the production of hypertension in rats.

A glance at Table III shows that the capacity of the resin for binding fecal sodium increased as the dietary sodium concentration increased. However, the percentage of ingested sodium excreted in the feces varied inversely with the sodium content of the diet. The very high sodium diet contained nearly ten times as much sodium as did the "medium" and "high" sodium diets. With the ingestion of such a high dietary intake of sodium the capacity of the resin increased to 1.43 meq. per gram. Although the percentage of dietary sodium excreted in the feces increased from 1.2 per cent on the diet without resin to 12.0 per cent on the diet plus resin, there was still a large amount of sodium absorbed. This absorption of sodium was reflected in the urinary

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excretion of sodium which decreased by about 50 per cent, but remained seven to ten times as great as on the "medium" sodium diet.

DISCUSSION

The practice of feeding ion exchange resins to rats in an attempt to evaluate their usefulness in man is a helpful, but far from conclusive test. It has been shown, in man, that the addition of an anion exchange resin to the cation resins enhances the sodium binding capacity of the cation resins by 20 to 30 per cent (Martz, Kohlstaedt, and Helmer[7]). However, in rats there was little or no enhancement of the capacity of Amberlite XE-64 when the anion exchanger Amberlite XE-58 was added. The reason for this difference between man and rat has not been investigated, but since it does exist, it is necessary to be wary of transferring rat results to the clinic. Fortunately for the clinician the difference in this case favors man.

From the study of several carboxylic and sulfonic acid resins it is clear that there is a wide variation in the sodium binding capacity of resins of each type. The sodium uptake per gram of resin varied for the carboxylic acid resins from 0.38 to 0.88 meq. per gram and for the sulfonic acid resins from 0.12 to 0.44 meq. per gram. None of the sulfonic acid resins tested had as high a sodium binding capacity as the best carboxylic acid resins, and only one, Dowex 50W, was as effective as the standard carboxylic acid resin, Amberlite XE-64, in the in vivo uptake of sodium. Unless other sulfonic acid resins can be developed with higher in vivo capacities for binding sodium, it is not necessary to submit patients to the greater acidity of the sulfonic acid type resin in order to obtain maximum sodium binding.

Since the addition of anion resin does not affect the sodium binding capacity of the cation resins in rats, any testing of anion resins in these animals is open to question. In an attempt to find a resin that would bind chloride, two anion exchange resins were compared with Amberlite XE-58. In rats, neither Amberlite XE-114 nor Duolite A-41 increased the fecal chloride excretion. However, in man, Martz[12] found a slight increase in fecal excretion of chloride when Amberlite XE-114 was substituted for Amberlite XE-58 in the cation-anion mixture. Again in these experiments the rat was not affected by changes which did alter the fecal excretion of ions in man. The results obtained with cation exchange resins in rats correlate well with those reported for the use of these same resins in man, but there is a lack of correlation between man and rat when the anion exchange resins are tested in both species.

The effect of dietary sodium on the efficiency and sodium binding capacity of carboxylic acid resins is shown clearly when the result obtained in rats on a sodium deficient diet is compared with that on the standard diet, containing 35 times as much sodium. When the sodium concentration of the diet was low the measured sodium binding capacity of the resin was low, but the percentage of ingested sodium that was excreted in the feces was high. The effect of dietary sodium level on the sodium binding capacity of ion exchange resins has been pointed out previously by Ch'en and Freeman.[13] This finding, which has been amply confirmed by our experiments with diets containing a wide range

of sodium concentration, serves to emphasize the necessity of keeping the sodium intake low if there is a desire to decrease the absorption of sodium from the gastrointestinal tract.

SUMMARY

Two carboxylic acid exchange resins, Amberlite XE-112 and Amberlite XE-103, were found to have two to three times the sodium binding capacity of Amberlite XE-64 in in vivo experiments in rats.

None of the sulfonic acid cation exchange resins tested was any more efficient in in vivo binding of sodium than was Amberlite XE-64.

The addition of anion to the cation exchange resin fed rats did not enhance the sodium binding ability of the cation resin.

Two phosphonic acid cation exchange resins were less than one-half as efficient as Amberlite XE-64 in binding sodium in the gastrointestinal tract of the rat.

The higher the dietary intake of sodium the greater was the amount of sodium bound per gram of resin, but the lower was the percentage of ingested resin that was excreted in the feces.

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STUDIES ON COCCIDIOIDES IMMITIS: MORPHOLOGY AND SPORULATION CAPACITY OF FORTY-SEVEN STRAINS

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ALTHOUGH the descriptions of *Coccidioides immitis* found in the literature indicate considerable variation in the morphology of this fungus (Baker, Mrak, and Smith[1]), the degree of this variation has not received sufficient emphasis. During the course of other studies in this laboratory, several aberrant strains were encountered, the morphology of which on Sabouraud's dextrose agar would not permit even a hint as to the identification. The attendant difficulties, especially in a diagnostic laboratory, were immediately apparent, and accordingly, a systematic study of a variety of strains was initiated. The results are herein presented.

METHODS

All strains mentioned in this paper were isolated from human cases of coccidioidomycosis with the exception of three, two of which were isolated from soil and one from a dog.† For specific verification, each of 47 strains was inoculated intraperitoneally into mice. This animal was selected in preference to the guinea pig because of the seemingly more uniform susceptibility. All animals died, were autopsied, and microscopic preparations of the lesions examined. Positive identification was indicated by the presence of spherules containing endospores. Each strain was then cultured on two slants of Sabouraud's dextrose agar and two slants of 1 per cent glucose agar enriched with 0.5 per cent yeast extract. The cultures were incubated at 34° C. and observed for either thirty or forty-five days. At the end of this time lactophenol cotton blue preparations were examined microscopically. The colonial and microscopic morphologies were recorded. In addition, one strain, "Herreth," which was known to be nonspore bearing, was maintained through 3 passages in mice, isolated by cultivation on malt agar, and then cultured and observed in the same manner as just described. For purposes of illustration, 21-day-old Petri dish giant colonies were prepared using Sabouraud's dextrose agar (Figs. 1 and 2). Observations of a few strains on other media are included. Also included are casual observations made during the course of other experimental investigation.

RESULTS

No strain differences could be detected through examination of spherules found in the tissue phase produced in infected mice. Fig. 3, however, adds

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confirmation to the observation recorded by Conant and associates[2], that immature spherules may bear a marked resemblance to the tissue phase of other pathogenic fungi.

The gross morphology of most strains conformed to the descriptions given by Conant and co-workers,[2] "the organism develops at first as a moist membranous colony which appears to be closely applied to the surface of the agar.

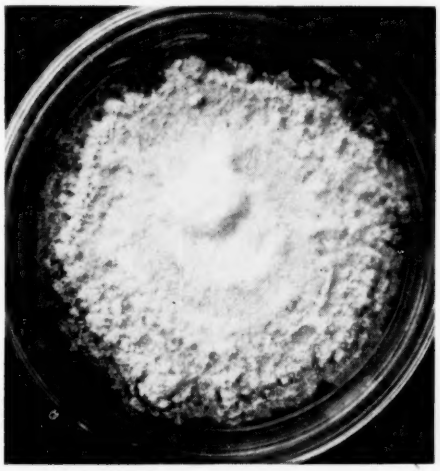

Fig. 1.—Three-week-old culture of *C. immitis*, King strain, on Sabouraud's dextrose agar.

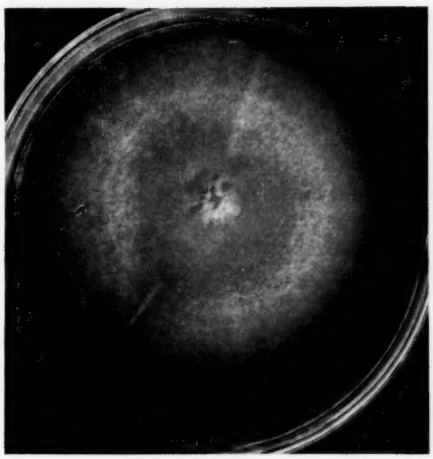

Fig. 2.—Three-week-old culture of *C. immitis*, Herreth strain, on Sabouraud's dextrose agar.

However, the organism quickly develops an abundant cottony aerial mycelium which is white at first but becomes tan to brown with age." Although the groupings are entirely artificial, the variations encountered did permit a rough arrangement into the following types: (a) Typical white or tan mycelium relatively sparse to abundant in quantity; (b) white, knotty, rough,

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adherent, yet dry; (c) strikingly colored, beige to dark brown; (d) flat and wet, with or without coremia which gave a spiculed appearance; (e) luxurious mycelium, distinctly yellow.

The classical morphology is typified by the giant colony shown in Fig. 1. An extreme variation, however, is that shown by a different strain, on the same medium (Fig. 2), the morphology of which places it in type 4. This strain, even after mouse passage, never produced more than a sparse fuzz of mycelium. As would be expected, there was considerable overlapping because often a single strain fell into one or more of the type classifications depending upon the culture medium used and, of course, the age. For example, the Mrak strain develops a yellow color only on glucose yeast extract agar.

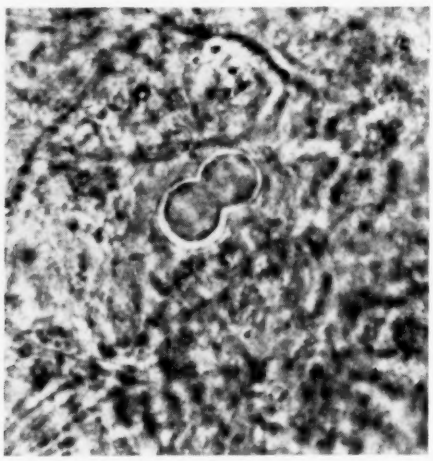

Fig. 3.—Microscopic preparation of a mouse lung showing *C. immitis* spherules resembling *Blastomyces dermititidis.* (×950.)

Another strain, incidentally, displays this characteristic when cultured on malt extract agar. Yet another strain produces luxuriant white aerial mycelium on Sabouraud's medium but fits readily into type 2 when grown on glucose yeast extract agar. Of the 47 strains studied, on one or more media, 6 strains fell into type 2, 7 into type 3, 3 into type 4, 2 into type 5, and the remaining strains into type 1.

Although there appeared to be no correlation with colonial morphology, microscopic morphologies were found to be equally variable, and often a single strain was found to present entirely different morphologies depending upon the culture media used. The classical morphology of *C. immitis,* showing free, or chains of, rectangular to ellipsoidal arthrospores (about 3 by 5 microns) (Figs. 4*A* and 5) was represented by the majority of strains, but considerable deviation in both quantity and quality of such structures was observed. Typical of the extremes in variation are those strains pictured by Figs. 6 and 7. Spores may be rectangular, ellipsoid, or spherical, as described by Conant and collaborators,[2] or may present grotesque shapes produced by

variations in size and shape of adjoining cells. In fact, it becomes difficult to draw the line between true spores and fragments of mycelium such as those shown in Fig. 7. The median size of this type of spore is 2.5 by 11 to 2.5 by 13 microns, but the range extends from 2.2 by 5 to 2.5 by 24.5 microns. Certainly, the differentiation could not be made entirely upon the thickness of the wall, because many strains produced cells which were not thick-walled yet conformed to the typical spore in all other respects. Cells such as those

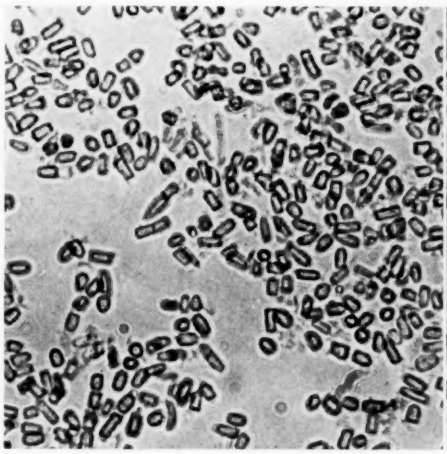

Fig. 4*A*.

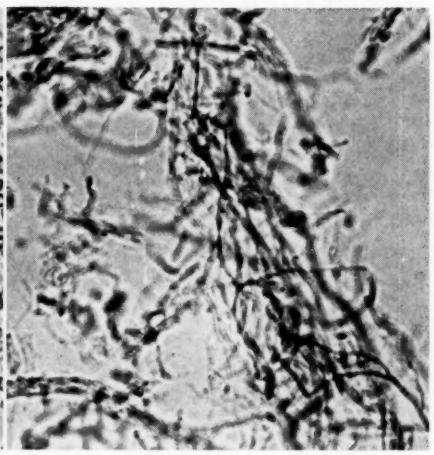

Fig. 4*B*.

Fig. 5.

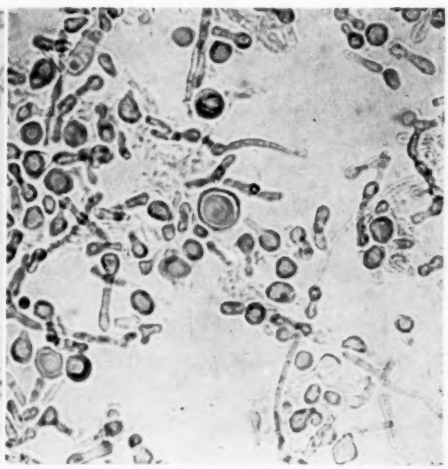

Fig. 6.

Figs. 4 to 7.—Microscopic preparations of four-week-old cultures of *C. immitis*. (×440.)
Fig. 4*A*.—Bruce strain on glucose yeast extract agar.
Fig. 4*B*.—Bruce strain on Sabouraud's dextrose agar.
Fig. 5.—Silveira strain on glucose yeast extract agar.
Fig. 6.—Warren strain on Sabouraud's dextrose agar.

shown in Fig. 6 seemed to consist mostly of spheres measuring 5.5 to 6.5 microns in diameter; yet diameters of 12 microns were not infrequent. The smallest cells observed among all strains were spheres 2.2 microns in diameter.

Twenty-one of the strains produced typical spores, in varying quantities, on both of the media used. Nineteen of these produced nothing but typical spores; two were mixed with atypical. Seven strains produced only atypical spores and did so on both media. Three strains produced typical spores on the yeast extract medium and only atypical spores on the Sabouraud's. Conversely, three other strains produced typical spores on the Sabouraud's medium

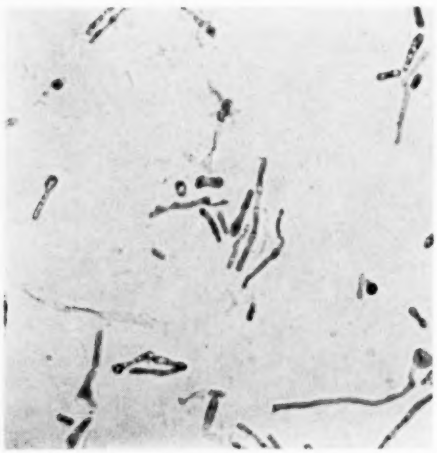

Fig. 7.—46× strain on Sabouraud's dextrose agar.

and atypical spores on the yeast extract agar. Four strains produced spores on the yeast extract medium only, and these were all atypical. Six other strains produced spores only on the yeast extract medium, but these were all typical spores. Three strains were nonspore formers on either medium. These results are tabulated in Table I.

As for the quantities of spores, almost the entire mycelium of some strains converted into spores, and microscopic preparations revealed only these spores (Fig. 4*A*) whereas among other strains, spore formation was moderate to exceedingly rare or even absent. As shown in Table I, thirteen strains formed no spores on Sabouraud's medium, including the strain represented by Fig. 4*B*, but ten of these strains sporulated in varying quantity and morphologic type when cultured on the yeast extract agar (Figs. 4*A* and 4*B*). Three of the 13 strains, however, did not sporulate on either media. One of these strains has never been observed to produce spores on any of several kinds of media including Sabouraud's dextrose and the yeast extract agar (Fig. 2); yet the hyphae, when inoculated into mice, consistently produced spherules and death of the animals.

Another strain, a recent isolate submitted for routine identification, was not included in the systematic study but is worthy of mention. This culture, growing on the medium upon which it was received, was completely deceiving in

TABLE 1. SPORULATION OF 47 *C. immitis* STRAINS ON SABOURAUD'S DEXTROSE AGAR AND GLUCOSE YEAST EXTRACT AGAR (GYE)

TYPE OF SPORULATION	NUMBER OF STRAINS
Spores, typical* morphology, on both media	21
Spores, atypical morphology, on both media	7
Spores on both media, typical* morphology on Sabouraud's only	3
Spores on both media, typical morphology on GYE only	3
Spores on GYE media only	10
(atypical morphology)	(4)
(typical† morphology)	(6)
No spores on either	3

*Two of these strains produced an equal mixture of typical and atypical spores.
†One of these strains produced an equal mixture of typical and atypical spores.

appearance, and microscopic preparations revealed an absence of spores. Yet this strain sporulated readily when transferred to other media. Positive identification was by mouse inoculation.

Spores in seemingly greater quantities were more often found on the yeast extract medium; unfortunately this cannot be deemed infallible identification procedure inasmuch as two strains (including Fig. 6) produced many spores on Sabouraud's medium, but only rarely formed spores on the yeast extract. In no instance, however, were spores observed on Sabouraud's, and not also on the yeast extract.

DISCUSSION

Contrary to the general impression, these data indicate that *C. immitis* in culture is not a fungus which always sporulates readily, typically, and in astronomical quantities. Certainly, many strains do possess this characteristic and, consequently, still demand the respect usually accorded this organism. It seems necessary, however, to indicate the frequency of exception in order that more exacting diagnostic techniques may be instituted. The surprisingly large number of strains which did not sporulate, especially on the widely used mycological medium of Sabouraud's, or which produced only spores possessing an atypical morphology, indicates the need of close observation before eliminating the possibility of *C. immitis* identity. And often the presence of typical spores which would offer a clue is masked by tremendous numbers of bizarrely shaped elements. The observations recorded evince the importance of nutritional requirements, but obviously this is not the entire answer to sporulation. This does not explain why as many as 150 Petri dish cultures, inoculated with the same strain and under identical conditions, may yield as many as 15, for example, which sporulate poorly. The common observation that sporulation usually occurs most rapidly and in greater abundance on the drying edge of agar slants might indicate rigid humidity requirements. Unfortunately, neither of the two media studied proved entirely satisfactory for identification by sporulation, and only recourse to animal inoculation and the finding of endospore-filled spherules for identification remains. One must be ever cognizant of the possibility of confusing immature cells of the tissue phase with other fungi. The microscopic preparation in Fig. 3, showing a tissue phase of *C. immitis,* serves as an example of this potential confusion. This preparation was made from lung tissue of a mouse inoculated intraperitoneally with spores.

Examination of the case histories from which these strains were isolated evidenced no correlation between spore morphology and pathogenicity. Nevertheless, a more extensive study might yield interesting results.

SUMMARY

A morphologic comparison of 47 strains of *C. immitis* on two different media was undertaken. The variation in colonial and microscopic morphology was emphasized. Absence of sporulation on Sabouraud's dextrose agar was observed with 10 strains. Three strains produced no spores on either media. All strains killed mice, and all strains produced exactly the same type of spherules.

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HUMAN ESTERASES*

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THE literature on the specificity of esterases is so voluminous that its review within the framework of this study would be impossible. For detailed information the reader is referred to chapters in textbooks of enzymology. Suffice to say that, as a result of a large number of studies, a classification of these enzymes has emerged. In general, esterases are divided in two large classes, the aliesterases[1] which hydrolyze esters of N-free alcohols and phenols, and cholinesterases, with a substrate specificity implied in their name. This classification does not include some of the enzymes hydrolyzing esters of procaine,[2] tropins,[3] and various alkaloids on which the data available are relatively scanty. The aliesterases are again subdivided into esterases proper which have a substrate preference for short-chained esters of simple alcohols, and lipases which hydrolyze also long-chained esters of polyhydric alcohols (fats, oils) with great efficiency. The cholinesterases also fall into two subclasses, the so-called true (or specific) cholinesterases[4] which attack acetylcholine preferentially, and pseudo- (or nonspecific) cholinesterases which will hydrolyze almost any choline ester. The dividing lines between these groups of enzymes are not entirely sharp because of a multiple overlapping of substrate specificities and of effects of activation and inhibition by various substances. It would appear that the most clear-cut single distinctive feature between any two groups is the markedly different sensitivity of aliesterases and cholinesterases to eserine and similar alkaloids[4] to which cholinesterases are about a thousand times more sensitive than aliesterases. The experimental results on which this classification is based were obtained, almost without exception, by titrimetric or gasometric methods. The introduction of colorimetry into the study of esterases marks an important advance in the refinement of methods. The substrates used for colorimetric methods are either directly chromogenic (esters of nitrophenol), or the color is developed by azo coupling in a second step (phenolic and naphtholic substrates). A recent addition to the colorimetric methods is the adaptation[5] of Hestrin's hydroxamic acid technique.[6] This latter method is less sensitive than the previously mentioned ones, and, since it determines not the product of hydrolysis but the residual substrate, it is inaccurate in cases of a low percentage of hydrolysis.

In the present report a study on the specificity of human esterases will be presented. The results were obtained by the use of a large number of phenolic and naphtholic substrates.

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MATERIAL AND METHODS

The *enzymes* used were human serum, urine, and extracts of human pancreas and liver. The extracts were prepared by homogenizing fresh tissue with 9 volumes of cold saline in a Potter-Elvehjem glass homogenizer and repeated freezing and thawing of the homogenates. After three to four consecutive freezings and thawings the homogenates were centrifuged for ten minutes at about 2,500 r.p.m. and filtered, if necessary. In this way almost crystal-clear extracts were obtained, the activity of which was not inferior, and often superior, to that of the original homogenates. In the frozen state serum, urine, and the extracts could be kept for several months without loss of activity. At room temperature liver extract was relatively stable; however, pancreatic extract lost activity rapidly (sometimes as much as 90 per cent in twenty-four hours).

Some of the *substrates* used were commercial preparations; most of them, however, were synthesized by esterification of phenol or naphthol with the corresponding acyl chloride or anhydride in an acetone-pyridine medium. When the acid chlorides were not available commercially, Einhorn and Hollandt's acid-phosgene method[7] or the thionyl chloride procedure as described by Nachlas and Seligman[8] was employed. In addition to these conventional procedures, one more method of esterification was found to yield excellent results in that the esters obtained were remarkably free from colored condensation products. Even naphthyl acrylate could be prepared in good yield and high purity. The method is as follows:

The phenol or naphthol is dissolved in about 10 volumes of acetone, and about 110 per cent of the calculated amount of NaOH is added in the form of a 25 to 30 per cent solution. As a rule, the alkali will not dissolve completely but form a pool in the bottom of the flask. Distilled water is now added drop by drop, under continuous shaking, until the solution becomes homogeneous. The flask containing the alkaline solution is placed in ice water, and a slight excess of acyl chloride is added drop by drop, under stirring. Sodium chloride precipitates promptly. The mixture is allowed to stand at room temperature for fifteen minutes and decanted from the solid.

Benzoylated naphtholsulfonic acids were prepared by the method of Whitmore and Gebhart.[9] Regardless of the method of esterification, the esters were recovered by pouring the mixture of synthesis into cold water. The oily esters were purified by decolorization with charcoal and by shaking out their solutions in ether with NaOH. The solid esters were recrystallized twice from dilute acetone or methyl alcohol.

Buffered substrate solutions were made up as follows: First of all, approximately 0.03M stock solutions in acetone were prepared of the substrates. These stock solutions could be kept in the refrigerator for months without decomposition. For use, 1 ml. of stock solution was blown into 80 ml. of distilled water, or, in the case of highly insoluble substrates such as phenyl and naphthyl esters from C_8 up and all esters of naphthols of the AS type (anilids of hydroxynaphthoic or hydroxycarbazole carboxylic acids), into 80 ml. of a 30 per cent solution of propylene glycol. In this way clear solutions or somewhat opalescent suspensions of good stability were obtained of all substrates. The original reason for the incorporation of propylene glycol was the enhancement of solubility and stability; however, it was soon discovered that propylene glycol is also a powerful activator of all esterases. The degree of activation depends on the substrate and may range from zero or slight inhibition (as in the case of the acetates of α- and β-naphthol) to over 1000 per cent (long-chained esters). The activity of the enzymes in the presence of taurocholate is only slightly affected by propylene glycol. Twenty milliliters of a 0.2M phosphate buffer of pH 6.5 to 7.4, depending on the case (see later), was added to the solutions. The buffered substrates could be kept in the icebox for several days with only such minimal decomposition as to cause no trouble in the determination of enzymatic activity.

The phenol or naphthol liberated was determined by azo coupling. The efficient range was about 0.015 to 0.20 micromole per sample (1.5 to 50 micrograms, depending on

the molecular weight of the phenolic substance). With phenol or naphthol produced within the range mentioned, hydrolysis was proportional with both the amount of enzyme and the length of time of incubation up to three hours. In the case of phenol, the method described previously[10] was followed. In the case of naphthols the procedure had to be modified because of the excessive insolubility of naphtholic azo dyes. It was found that the tedious and time-consuming extraction with ethyl acetate can be avoided since most azo dyes may be kept in a perfectly clear colloidal solution by the use of detergents. Especially Brij 35, a polyethylene glycol lauryl alcohol, made by the Atlas Powder Co., and sodium lauryl sulfate proved to be very efficient. The latter had the added advantage of stopping the enzymatic reaction immediately.

The choice of the diazonium salt depended on the phenolic component of the substrate. A number of commercially available diazonium salts were found which gave excellent, dark shades, stable over long periods of time. For phenol, Red B Salt (diazotized 5-nitro-2-amino-anisole; shade, orange-red); for α-naphthol, Red ITR Salt* (diazotized 4-diethylsulfonamido-2-aminoanisole; shade, purple) or Fast Violet B Salt* (diazotized 6-benzoylamino-4-methoxy-*m*-toluidine; shade, greenish blue-gray with Brij 35; purplish with lauryl sulfate); for β-naphthol, Fast Violet B Salt (shade, cherry red) were selected. In the case of the AS naphthols the azo dyes formed with the commercial diazonium salts were too insoluble to yield a permanently clear solution. For this reason, 2-diazonaphthalene-6,8-disulfonic acid was prepared according to the procedure given by Venkataraman.[11] This diazonium salt yielded clear, brilliant reddish solutions with the AS naphthols. The wave lengths at which the solutions were read are as follows:

phenol-Red B Salt	490 mμ;
α-naphthol-Red ITR Salt	555 mμ;
α-naphthol-Fast Violet B Salt-Brij	610 mμ;
β-naphthol-Fast Violet B Salt-lauryl sulfate	570 mμ;
β-naphthol-Fast Violet B Salt	510 mμ;
Naphthol AS-diazonaphthalenedisulfonic acid	525 mμ.

Taurocholate, used as an inhibitor of esterase and as an activator of lipase, will shift the shade of some of the azo dyes somewhat toward the longer wave lengths. Whenever taurocholate is used and high accuracy is desired, two standardization curves should be prepared; one without and one with the normally employed amount of taurocholate added to the samples.

The method of determination of enzymatic activity was the following:

Five milliliter samples of buffered substrate were incubated with 1 ml. of enzyme dilution for one hour at 37° C. The amount of enzyme used depended on the substrate. With α-naphthyl butyrate, the amounts used were liver, 5 μg; pancreas, 5 to 10 μg; serum, 1 ml. of a 1:1,000 or 1:2,000 dilution; urine, 1 ml. of a 1:10 or 1:20 dilution. Controls were incubated without enzyme. At the end of one hour the rack holding the test tubes was placed in cold water, and 1 ml. of enzyme was added to the controls. About 100 mg. of diazonium salt was dissolved in 6 to 7 ml. of water and 13 to 15 ml. of a 5 per cent solution of either Brij 35 or of sodium lauryl sulfate was added to the solution (neither the amount of diazonium salt nor that of the detergent are very critical, and the solutions are stable for several hours). One ml. of this diazonium salt solution was added to each of the tubes which then were inverted twice and allowed to stand. They were read after ten to thirty minutes' standing against their controls at the appropriate wave length. The α-naphthol-Fast Violet B Salt-lauryl sulfate combination was an exception because it changed its shade from the initial red-brown to the final purple rather gradually. It was read between twenty-five and forty minutes.

Table I shows the pH dependence of the rate of hydrolysis, with α-naphthyl butyrate as a substrate. The relatively high pH optimum of serum is clearly indicated. Most experiments were run at the compromise pH value of 7.2 to 7.4, except in the case of naphthol AS acetate as substrate. This ester is somewhat unstable above pH 7, and, as

*Supplied by the General Dyestuff Corporation.

preliminary experiments have shown, is hydrolyzed by enzymes optimally around pH 6.3 to 6.5. For this reason, whenever the substrate was naphthol AS acetate, the pH was kept within the limits mentioned.

TABLE I. THE pH-DEPENDENCE OF THE RATE OF HYDROLYSIS OF α-NAPHTHYL BUTYRATE BY HUMAN ESTERASES

ENZYME	pH					
	6.2	6.6	7.0	7.3	7.6	8.0
Liver	19	22	23	23	22	20
Pancreas	38	52	48	38	33	28
Serum	13	19	29.5	34	40	39

RESULTS

The rates of hydrolysis for a number of esters of phenol, α- and β-naphthol and of naphthol AS acetate are given in Table II. The reason why such un-

TABLE II. RELATIVE RATES OF HYDROLYSIS OF PHENOLIC AND NAPHTHOLIC ESTERS BY HUMAN ENZYMES

SUBSTRATE	SYMBOL IN TEXT	LIVER		PANCREAS		SERUM		URINE	
		O	TCh	O	TCh	O	TCh	O	TCh
Phenyl butyrate	φ 4	620	150	650	1150	850	650	—	—
Phenyl undecylenate	φ 11 △	450	120	170	1600	15	9	—	—
Phenyl laurate		130	60	47	400	1	1	—	—
Phenyl benzoate	φ benz.	550	12	23	40	60	42	—	—
α-Naphthyl acetate	α 2	260	2	270	850	700	300	—	—
α-Naphthyl propionate	α 3	580	50	400	1000	950	350	—	—
α-Naphthyl acrylate		160	55	700	2800	—	—	—	—
α-Naphthyl butyrate	α 4	1000 (1300)	180	1000 (1400)	2200	1000	260-700	1000	730
α-Naphthyl valerate		850 (2300)	220	400 (1020)	1450	750	390	500	450
α-Naphthyl trimethyl-acetate		16	2	< 2	< 2	14	10	4	2
α-Naphthyl caproate	α 6	120 (240)	70	150 (150)	720	550	140	950	580
α-Naphthyl isocapro-ate		450	150	55	920	300	90	170	220
α-Naphthyl heptoate		330 (1100)	140	220 (350)	1200	—	—	—	—
α-Naphthyl caprylate		230	100	560	3400	—	—	—	—
α-Naphthyl caprate		60	40	170	2300	—	—	—	—
α-Naphthyl unde-cylenate	α 11△	42	28	140	2000	1	0.8	20	470
α-Naphthyl laurate		8	6	55	1500	< 0.3	< 0.1	13	300
α-Naphthyl myristate		±2	±1.5	13	1100	< 0.1	< 0.1	5	215
α-Naphthyl benzoate		48	—	104	—	—	—	—	—
β-Naphthyl acetate	β 2	17	1	230	1050	400	250	—	—
β-Naphthyl pro-pionate	β 3	78	17	720	1400	610	370	—	—
β-Naphthyl butyrate	β 4	460	110	960	2600	460	300	1200	820
β-Naphthyl unde-cylenate	β 11△	22	17	230	650	4	1.3	—	—
β-Naphthyl laurate		9	5	32	1300	0.7	0.15	10	260
β-Naphthyl myristate		2.5	1.5	9	600	—	—	—	—
β-Naphthyl benzoate		40	—	38	—	—	—	—	—
Naphthol AS acetate		9	3.5	75	140	12	5	16	20

All substrate mixtures from C_8 up and in the case of naphthol AS acetate contain 25 per cent propylene glycol.

— = not examined.
O = without taurocholate.
TCh = in the presence of 0.004M taurocholate.
Data in parentheses: activity in the presence of 25 per cent propylene glycol.

usual substrates as acrylic and undecylenic esters were included in the series is biochemical[12] and histochemical[13] findings according to which unsaturated esters are relatively specific substrates for pancreatic lipase. Naphthol AS acetate was studied because the enzyme or enzymes responsible for its hydrolysis appear to be different, at least in some tissues, from esterases which attack esters of α- and β-naphthol.[14] According to Benditt and French (unpublished), crystalline chymotrypsin hydrolyzes naphthol AS acetate at a high rate, while it does not touch esters of the simple naphthols.

The data represent averages of at least 3 different samples of each individual enzyme. While the absolute activities of the enzymes showed a rather wide scatter (a range of about fivefold), the relative rates of hydrolysis for the different substrates were surprisingly uniform in the case of each individual type of enzyme. This finding indicates that the major part of the activity in each case was due to a single enzyme (or to a group of closely related enzymes), and the admixture of enzymes of other types was either of a minor quantitative importance or of relatively constant ratio. The variations seldom exceeded 25 per cent up or down, except in the case of serum. Individual, presumably normal, sera showed considerable departures from the data given for pooled serum. Hepatic and pancreatic extracts had a tendency to develop their distinctive features (preference for short-chained substrates and inhibition by taurocholate in the case of liver; relatively high rates of hydrolysis for long-chained substrates, and activation by taurocholate in the case of pancreas) on prolonged storage, beyond the degree observed in fresh extracts.

For easier survey and comparison, the rate of hydrolysis of α 4 in the absence of taurocholate is assigned the arbitrary value of 1,000. The ratios between esters of phenol, α- and β-naphthol, and naphthol AS are given as molar ratios. The absolute rates of hydrolysis of α 4 by the various enzymes were found to be (per mg. of tissue or per milliliter of serum and urine per hour) 28 to 130 μM for liver; 20 to 66 μM for pancreas; 90 to 250 μM for serum, and 4 to 18 μM for urine.

The data given in Table II show a number of interesting features, some of which have been known for a long time; others, however, have not been reported before. Attention is called, among others, to the following: (a) Butyrate is hydrolyzed at the highest rate by all enzymes. The only exception is the hydrolysis of β 3 by serum at a rate higher than the corresponding butyrate. The hepatic enzyme shows a second peak at C_7, pancreas at C_8, and urine at C_6. (b) With the exceptions mentioned, the rate of hydrolysis rapidly drops with increasing chain length. This decrease in rate is far more marked in the case of serum than with the other enzymes. (c) Trimethylacetate is an extremely poor substrate for all enzymes. (d) n-Caproate is hydrolyzed faster than isocaproate by pancreas, serum, and urine; the reverse holds true for liver. This preference for iso-chains by hepatic enzyme has been described by Terroine.[12] (e) In disagreement with histochemical findings[13] obtained by the use of a different series of substrates, the pancreatic enzyme shows no marked preference for unsaturated esters. (f) Naphtholic esters are hydrolyzed by pancreas at highly varying rates; however, in the presence of taurocholate the

rates become much more uniform. (g) The dependence of the rate of hydrolysis on the chain length of the fatty acid is markedly affected by the alcoholic moiety (compare the ratio φ 4/φ 11 Δ *vs.* β 4/β 11 Δ in the case of liver). (h) Liver and serum hydrolyze esters of α-naphthol much more efficiently than the corresponding β-isomers. Pancreas and urine are indifferent in this respect.

The marked effect of the alcoholic moiety on the rates of hydrolysis by the various enzymes has prompted further studies with the use of esters of more complex phenols and naphthols. The data given in Table III are only approximate because no separate calibration curves were made with the various phenols and naphthols. The colorimetric readings were taken in terms of the parent compound (phenol, α- and β-naphthol and naphthol AS). Only one sample of each tissue was tested in these experiments.

TABLE III. RELATIVE RATES OF HYDROLYSIS OF ESTERS OF COMPLEX PHENOLS AND NAPHTHOLS BY HUMAN LIVER AND PANCREAS

SUBSTRATE	LIVER	PANCREAS
o-acetamidophenol benzoate	42	22
m-acetamidophenol benzoate	47	14
p-acetamidophenol benzoate	< 0.5	< 0.5
m-acetoxybenzoic acid	< 0.5	1
p-acetoxybenzoic acid	< 0.5	3
m-acetoxybenzoic anilide	< 10	9
p-acetoxybenzoic anilide	< 0.5	< 0.5
p-acetoxyphenylarsonic acid	< 0.5	0.8
5-acetamido-α-naphthyl acetate	13	10
7-acetamido-α-naphthyl acetate	300	28
1-benzoyloxynaphthalene-4-sulfonic acid	2.5	8
3-acetamido-β-naphthyl acetate	decomposes spontaneously	
5-acetamido-β-naphthyl acetate	1	25
7-acetamido-β-naphthyl acetate	4	36
8-acetamido-β-naphthyl acetate	< 0.5	2.8
2-benzoyloxynaphthalene-6-sulfonic acid	< 0.5	15
2-acetoxy-3-naphthoic acid (pH 6.5)	< 0.5	1
6-bromo-β-naphthol acetate	2	63
Naphthol AS (2-hydroxy-3-naphthoic anilide) acetate	10	75
1-acetoxy-2-naphthoic anilide	8	0
Naphthol AS-GR (2-hydroxy-3-anthroic anilide) acetate	< 0.5	< 0.5
Naphthol AS-LB (2-hydroxycarbazole-3-carboxylic chloroanilide) acetate	1	4

Rate for α-naphthyl butyrate = 1,000.

Attention is called to the following interesting points: (a) Phenols and naphthols containing a free carboxylic group are very poorly hydrolyzed or not at all. In general, the corresponding anilides are much better substrates. (b) In the case of the acetamido-phenols and naphthols, the position of the substituting group profoundly affects the rate of hydrolysis. (c) As in the case of the simple naphtholic esters, liver shows a marked preference for the α-isomers. Pancreas is more specific for substituted β-naphthols.

DISCUSSION

Before going into the discussion of the results, it should be made clear that the data presented apply exclusively to human enzymes. The pattern of the relative rates of hydrolysis (the so-called enzymatic profile) may be entirely

different in other species, and is known to be so in the dog. For instance, the statement of Ravin, Zacks, and Seligman,[15] according to which dog liver hydrolyzes β 2 at a higher rate than α 2, could be confirmed in our experiments. For this reason, the comparison of enzymatic profiles of other species, obtained with substrates containing a different alcoholic moiety, with those shown in the present paper is not necessarily meaningful.

There are a number of studies available on the pattern of hydrolysis of fatty acid esters by various enzymes. Only specific statements will be mentioned here. Terroine[12] found that among triglycerides the laurate, and among ethyl esters the butyrate is hydrolyzed most efficiently by pancreas. Triolein is attacked much faster than tristearin. Schønheyder and Volqvartz,[16] on the other hand, say that tributyrin shows the highest rate among triglycerides, both with liver and pancreas. Hofstee[17] studied the hydrolysis of acyl-salicylic acids by pancreas and found that the highest rate occurs between C_4 and C_7. According to Doyon and Morel,[18] horse serum hydrolyzes ethyl esters in the following decreasing order: caproate, butyrate, acetate, propionate. Huggins and Lapides[19] maintain that, in the case of *p*-nitrophenyl esters, human serum attacks the acetate, propionate, isobutyrate, and valerate at about equal maximum rates; n-butyrate is hydrolyzed somewhat slower, and trimethylacetate very much slower. Rat serum, on the other hand, shows a different pattern in that n-butyrate and valerate are split at the highest rates. Huggins and Moulton[20] find, with the use of the same substrates, that rat liver shows a maximum rate at C_3, and rat pancreas at C_5.

From the data in Table II it is at once obvious that not only pancreatic "lipase" and hepatic "esterase" are different enzymatic entities but that the enzyme of serum is also a distinctly specific enzyme. The origin and nature of serum esterase have been debated for over thirty years. It appears that species differences are very marked.[21] In some species the enzyme of serum resembles the hepatic type, in others, a mixture of variable proportions of the hepatic and pancreatic types. It is queer, however, that even the "pancreatic" enzyme of the blood does not seem to derive from the pancreas exclusively since a rather high level of activity persists after total pancreatectomy.[22] While it is possible that the human serum does contain relatively small amounts of hepatic esterase and traces of pancreatic lipase,[23] some of its features (exceedingly low rates of hydrolysis from α 11 Δ up; relatively high rate for α 6; β 3 hydrolyzed faster than β 4) cannot be explained in terms of these two enzymes. An important clue was secured in the present experiments when it was found that the hydrolysis of α 2 and α 4 by serum is suppressed by 10^{-5}M eserine to the extent of 96 per cent or more; that of φ benz., 90 per cent. Activity toward α 11 Δ is inhibited 55 per cent. Hydrolysis of the β-esters is inhibited less markedly: β 2, 50 to 80 per cent; β 4, 72 to 85 per cent. The figures given for β 2 are in good agreement with the data of Ravin, Tsou, and Seligman.[21] Activity toward AS acetate is practically unaffected. In contrast, hepatic esterase is completely uninfluenced, and pancreatic lipase is inhibited less than 40 per cent by this concentration of

eserine. On the basis of these data it is reasonable to assume that the bulk of the enzyme responsible for the hydrolysis of short-chained α-esters is a cholinesterase. This is in complete agreement with previous findings obtained by the use of different substrates.[4, 25] Rider, Moeller, and DuBois[26] were the first to suggest the use of a noncholine substrate for the estimation of serum cholinesterase activity. It would appear that the simple azo-dye methods can supersede the more cumbersome gasometric and other methods using acetylcholine as a substrate. The use of eserine in a control tube can serve as a safeguard of specificity. According to Grob, Lilienthal, Harvey, and Jones,[27] normal human plasma splits about 180 μM of acetylcholine per milliliter and per hour; de la Huerga, Yesinick, and Popper[5] report readings of 140 to 280 μM. These values are essentially the same as those obtained with α 4 as a substrate.

Whether or not the same enzyme is responsible for the hydrolysis of both short- and long-chained substrates cannot be decided with certainty until reasonably pure fractions will be available, although a method of the kind proposed by Myers and Mendel[28] may help to solve the problem.

Seligman and Nachlas[29] have found that pancreatic lipase can be detected in abnormal sera by the finding of a taurocholate-activated enzyme. In the present studies no such activation could be observed in the sera of 4 patients who had chronic pancreatitis or carcinoma of the pancreas, and had markedly elevated blood amylase activity. The level of urinary excretion of lipase was also normal in all cases.

The profile of the urinary enzyme[30] is very similar to that of pancreatic lipase except for the slight inhibition (instead of activation) by taurocholate in the case of short-chained esters.

SUMMARY

A simplified azo-dye method for the quantitative determination of phenols and naphthols is described. The enzymatic profiles of human esterases of liver, pancreas, serum, and urine, obtained by the use of phenolic substrates, are presented. The specific features of these enzymes are discussed. Human serum esterase is identified as a cholinesterase.

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DEPRESSION OF COPROPORPHYRIN EXCRETION BY AMITHIOZONE

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DURING the course of a recent study of the hepatic toxicity of amithiozone in tuberculous patients,[1] coproporphyrin excretion was observed to be reduced irrespective of the presence or absence of liver dysfunction as represented by other liver function tests, particularly the bromsulfalein test. The present study was undertaken to evaluate serially the changes in coproporphyrin excretion attributable to amithiozone and to correlate them with similar changes in hemoglobin concentration.

METHODS

Case material, therapeutic regimen, variables for study.—Twelve patients with far-advanced pulmonary tuberculosis were selected for study. The total period of observation ranged from thirteen to sixty-one weeks, with an average of thirty-one weeks. Each patient received 200 mg. of amithiozone daily during the period of treatment which varied from four to thirty-five weeks (average eighteen weeks).

Serial measurements, often weekly, were made of hemoglobin concentration (Hb) and urine coproporphyrin excretion (UCP). Values at the start of therapy were recorded in all but one case. Urinary coproporphyrin was determined by an improved procedure[2] for which the normal range is 100 to 300 μg per day.[3]

TABLE I. HEMOGLOBIN AND COPROPORPHYRIN VALUES OBSERVED IN PATIENTS WITH MINIMAL, MODERATELY ADVANCED, AND FAR-ADVANCED TUBERCULOSIS

EXTENT OF TUBERCULOSIS	NO. OF CASES	Hb (GM. %)		UCP (μG/D)	
		MEAN	RANGE	MEAN	RANGE
Minimal	6	14.3	12.0-15.5	153	89-217
Moderately advanced	6	14.8	12.6-16.0	188	116-226
Far advanced	12	14.5	13.0-15.5	161	83-322

Values obtained for these two measures are unrelated to the severity of the tuberculosis, as indicated in Table I which presents hemoglobin and coproporphyrin values obtained on three small groups of cases with minimal, moderately advanced, and far-advanced tuberculosis, respectively. None of the means are significantly different.

The effects of the drug on coproporphyrin excretion were unrelated to the response of the tuberculosis to treatment. In most instances there was little change in the tuberculosis. There was no evidence of hemolysis. Renal function was checked serially and remained normal in each case. Amithiozone added to urine did not affect the coproporphyrin determination.

Analysis of the data.—In order to make use of all the information contributed by sequential data of this type, individual graphs were prepared showing the changes in

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hemoglobin concentration and coproporphyrin excretion with time. The left-hand ordinate of each graph records a scale of measurement in terms of the approximate level of the variable about which the individual appeared to fluctuate normally. This enables a comparison of relative changes in hemoglobin concentration and coproporphyrin excretion. The right-hand ordinate records scales in terms of actual hemoglobin and coproporphyrin values. Duration of therapy is indicated by a horizontal bar near the bottom of each graph, and various extraneous factors which might affect the results are indicated at the top of the graph where appropriate.

RESULTS AND INTERPRETATIONS

The serial changes in hemoglobin concentration and urinary coproporphyrin excretion in relation to therapy with amithiozone are portrayed for each individual in Figs. 1*A* and 1*B*. The depressant effect of the drug on coproporphyrin excretion and hemoglobin concentration is evident in each instance, and the changes in both variables parallel each other more or less. Certain summary observations are evident from the graphs.

The stable level of hemoglobin varied from 13.2 to 15.5 Gm. per cent. The median value was 14.2 Gm. per cent. The stable level for coproporphyrin excretion varied from 100 to 320 μg per day, and the median value was 150 μg per day. In most instances the decrease in coproporphyrin excretion was sooner than the decrease in hemoglobin concentration. The first appreciable depression of hemoglobin occurred in a median time of six weeks (range one to eight weeks). The first appreciable depression of coproporphyrin excretion occurred in a median time of three weeks (range one to five weeks). The maximum depression of hemoglobin occurred in five to thirty-five weeks (median ten weeks); of coproporphyrin excretion in three to twenty-four weeks (median eight weeks). The relative depression of coproporphyrin was far greater. Thus at its lowest point the hemoglobin concentration was 70 to 87 per cent (median 80 per cent) of the stable level. At its lowest point coproporphyrin excretion was 20 to 60 per cent (median 40 per cent) of the stable level.

The return to normal after discontinuing therapy was also somewhat more rapid with the coproporphyrin test. In a few cases the hemoglobin remained depressed at least several weeks after the coproporphyrin values returned to normal. Half the cases overshot their stable level of coproporphyrin excretion after the drug was withdrawn, representing what appears to be a rebound phenomenon. In two instances this was associated with a lobectomy performed shortly after stopping therapy.

Certain individuals displayed occasional unexplained fluctuations to excessively high values. In one instance a rise in coproporphyrin excretion was definitely related to an episode of acute alcoholism. We suspect this as a factor in certain of the other unexplained excessive fluctuations.

Marked physiologic fluctuations in coproporphyrin excretion were apparent from the serial studies. The contrast with hemoglobin values is particularly striking. Week-to-week fluctuations varied from 10 to 70 per cent of the stable levels. Despite such wide fluctuations the depressant effect of amithiozone was clearly evident.

COMMENT

Though many agents have been found to increase the excretion of coproporphyrin, with or without correlated hematopoietic changes,[4] none, other than irradiation, has been described heretofore which depresses coproporphyrin excretion except as a concomitant of compromised renal function. Schwartz and co-workers[5] demonstrated in dogs that uranium, strontium, and plutonium poisoning resulted in marked depression of coproporphyrin excretion. How-

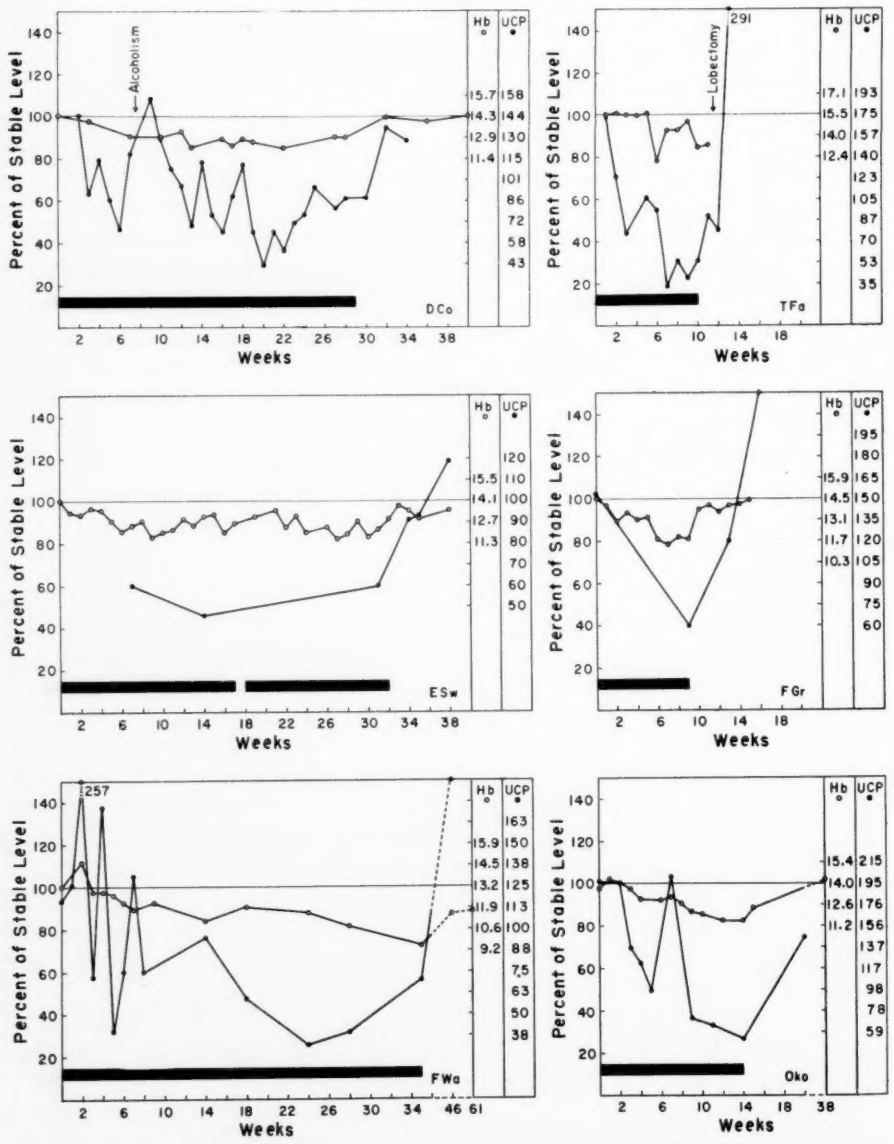

Fig. 1A.—Serial changes in blood hemoglobin concentration and urinary coproporphyrin excretion in relation to therapy with amithiozone. Horizontal bar indicates period of therapy.

ever, severe kidney destruction was also present. In another small group of dogs[5] total body irradiation in large doses resulted in depressed excretion of coproporphyrin until the preterminal phase. In these animals renal function was normal or increased during the period of coproporphyrin depression.[6] In contrast to these observations in dogs, Koch and associates[7] noted increased coproporphyrin excretion in six human beings receiving therapeutic doses of x-ray or radioactive iodine.

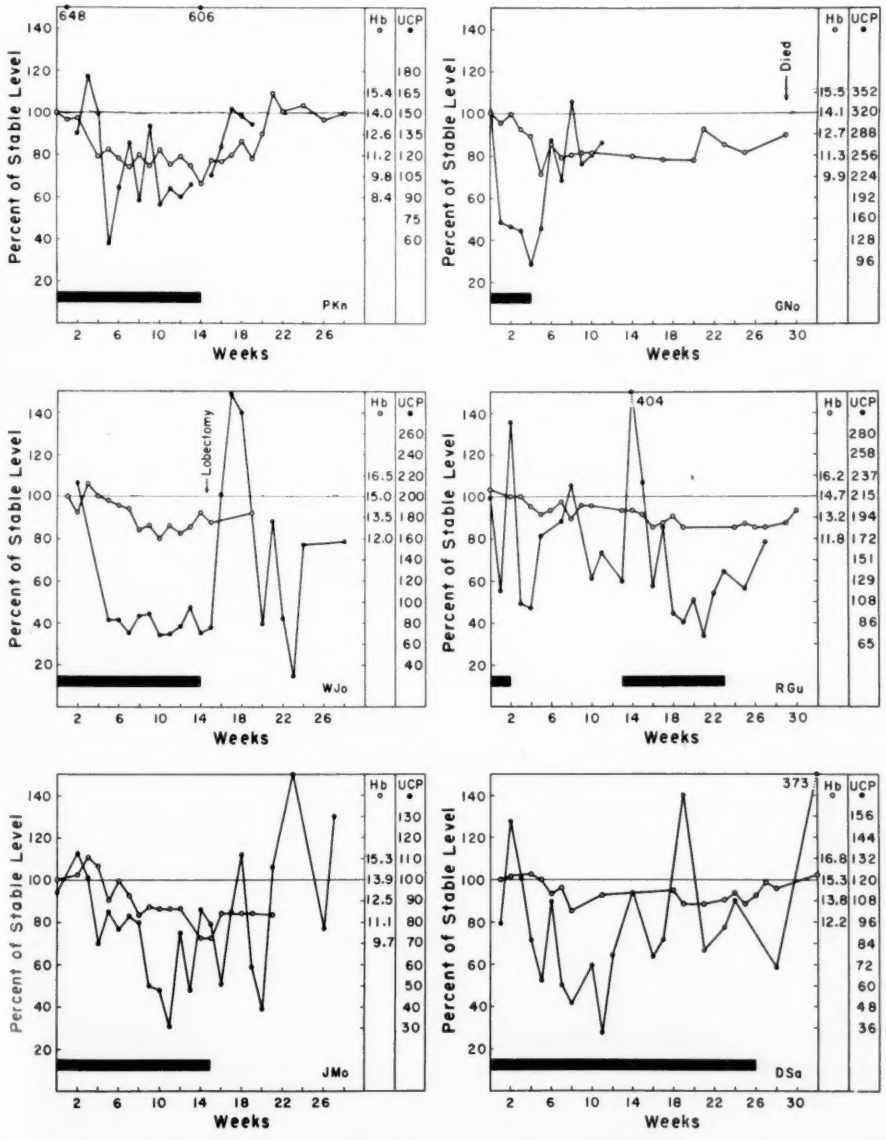

Fig. 1*B*.—Serial changes in blood hemoglobin concentration and urinary coproporphyrin excretion in relation to therapy with amithiozone.

The observed effect of amithiozone is probably on the hematopoietic system, since the changes in coproporphyrin excretion paralleled the changes in hemoglobin concentration and were unrelated to changes in bromsulfalein excretion. The use of amithiozone in tuberculosis is on the wane in this country after a brief trial. However, the observations recorded here suggest its potential value in the experimental study of hemoglobin metabolism.

SUMMARY

Amithiozone has been observed to depress coproporphyrin excretion without concomitant renal dysfunction. The blood hemoglobin concentration was depressed in parallel fashion, though its relative reduction was approximately half that of coproporphyrin excretion. After stopping the drug, coproporphyrin excretion returned to normal levels in two to six weeks. In approximately half the cases hemoglobin concentration remained depressed at least several weeks after coproporphyrin excretion returned to normal.

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THE EFFECTS OF MODIFIED HUMAN GLOBIN IN THE HUMAN SUBJECT

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PROTEIN is best given orally, but parenteral administration is often desirable and occasionally imperative. Furthermore, the oncotic properties of whole protein solutions render them valuable as plasma expanders for the treatment of shock and in certain types of hypoproteinemic edema. All available parenteral protein preparations demonstrate certain disadvantages. Amino acid solutions possess minimal oncotic value and appear to be utilized more for energy than for the repletion of protein stores during the catabolic phase of illness. Whole blood is the best plasma expander, but its relative scarcity and slow breakdown render it unsuitable for protein nutrition. The frequent contamination of plasma with the virus of homologous serum jaundice and the high cost of albumin inhibit general use of these proteins. Thus, any protein preparation which is suitable for parenteral use deserves careful study. This is particularly true of modified human globin which is prepared from red cells available in large amounts as a by-product of plasma manufacture.

This study concerns the administration of 355 units of globin to forty patients. Special emphasis was placed on its value in plasma protein repletion, 24 patients having been studied in this regard. Because of the problems attendant upon the use of any new parenteral preparation, considerable attention was given to toxic manifestions.

MATERIALS AND METHODS

The globin used in this study was prepared from human red cells by Sharpe and Dohme, Inc., and supplied in lyophilized form, each unit containing 16 Gm. Strumia and his associates[1-3] have described in detail the preparation, nature, and certain physiologic properties of this material. Globin was given as an 8 per cent solution in 2.5 per cent sodium chloride, 5 per cent glucose, or distilled water. In most instances two units (32 Gm.) were given in 400 ml. of distilled water, allowing two to four hours for infusion. Most subjects received 32 Gm. of globin each day for four to six days.

Globin in blood, urine, and serous effusions was measured by the Kendell-Abell method as modified by Sample, Strumia, and Satta.[4] This method was standardized against modified human globin added to the substance being tested. A satisfactory calibration curve was obtained, which gave a straight line passing just above zero, using a Klett-Summerson photoelectric colorimeter and a 420 μ filter.

Total serum protein was determined by the standard Kjeldahl method, correction being made for the serum nonprotein nitrogen. Serum albumin and globulin were measured by the method of Pillemer and Hutchinson.[5] Control determinations were obtained im-

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mediately preceding the administration of globin, and a number of samples were analyzed during treatment. The final specimen was obtained two days after treatment was stopped, and calculations with respect to total change were based on this and the control samples. Blood hematocrit was measured by the Wintrobe method and plasma volume by use of a standard T-1824 technique. Blood polypeptides were measured by the technique of Godfried.[6] Standard procedures were used for the determination of urinary nitrogen and nonprotein nitrogen; urinary protein was estimated from the difference between these values.

RESULTS

Toxic Reactions.—During the early portion of the study 12 per cent of the patients manifested toxic reactions to the infusion of globin in the form of fever, erythema, sweating, and tachycardia. Certain lots of globin appeared to be responsible for these manifestations, but speed of the infusion also exerted a strong influence on their occurrence. Even with lots of globin which consistently caused toxicity, symptoms were prevented or markedly ameliorated by slowing the rate of infusion. Only two extremely mild reactions have been noted with the use of the purified and more carefully controlled globin preparations available for the latter part of the study. Elevation of serum nonprotein nitrogen occurred in a number of instances, but values returned to normal after discontinuance of treatment except in those patients with chronic renal disease or terminal illness. Study of the urinary sediment by routine methods revealed no change during treatment. Proteinuria in amounts rarely exceeding 3 Gm. per day, determined by the method of Esbach, was noted uniformly during globin therapy. The proteinuria disappeared soon after cessation of treatment, and can be accounted for by the excretion of globin which reacts positively to the test employed. The degree of proteinuria, estimated by subtraction of urinary nonprotein nitrogen from total nitrogen, agreed within the limits of the accuracy of the globin determination, shown in Table I. Thus there was no indication that globin inhibited tubular reabsorption of natural proteins. Much poorer correlation was demonstrable in cases of preexistent albuminuria, and, although measurement of urinary globin is rather unsatisfactory in the albuminuric patient, it is possible that globin may increase proteinuria in the nephrotic subject.

After an informal report of the occurrence of increased red cells in the urine during globin therapy,[7] as demonstrated by the Addis count technique, this method of study was applied to three patients undergoing therapy (Table II). One patient with mildly active glomerulonephritis had increased red cells,

TABLE I. COMPARISON OF PROTEINURIA WITH GLOBINURIA

Globin dose (Gm./day)	0	16	32	32	32	32	32	0	0
Total urinary nitrogen (Gm.)	6.4	5.9	10.0	11.1	11.0	12.0	12.3	10.3	9.4
Urinary NPN (Gm.)	6.4	5.0	9.3	10.1	10.1	11.3	11.4	10.3	9.4
Estimated urinary protein* (Gm.)	0	6.8	5.3	6.8	6.8	5.3	6.8	0	0
Urinary globin (Gm.)	0	8.4	6.3	7.7	8.8	6.7	7.5	0.7	0

*Nitrogen ×7.5.

casts, and white cells prior to therapy. Severe flank pain occurred during two days of globin treatment, and two days later a marked increase in red cells and casts was noted. In a patient with no evidence of renal disease, a moderate increase in red cells and casts occurred during therapy. In the third patient, also free of evidence of renal disease, there was no significant change in the formed elements of the urine. Two patients with terminal carcinoma were given 4 and 6 units of globin, respectively, a few days before death. In neither instance were there any significant histologic changes in the kidney which could be attributed to the globin therapy.

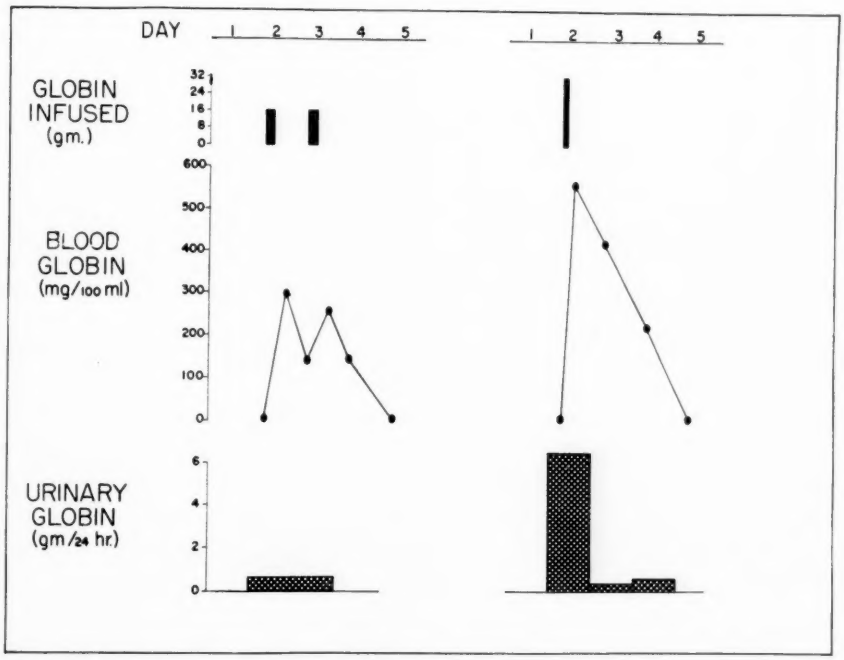

Fig. 1.—Blood and urinary globin following its infusion.

Serum and Urinary Globin.—The effect of the infusion of 16 Gm. of globin for two successive days on blood and urine globin levels is shown in Fig. 1. Blood levels were between 140 and 300 mg. per 100 ml. of blood, and, during the forty-eight hour period, approximately 4.5 per cent of the administered globin was excreted in the urine. When 32 Gm. of globin was infused more rapidly, the blood globin level rose to 560 mg. two hours after the infusion, decreasing to 420 mg., and then to 220 mg. during the next two days. Urinary excretion of globin amounted to 6.5 Gm., 0.4 Gm., and 0.6 Gm. during these three days, total urinary excretion during this period thus amounting to 23.5 per cent of the administered dose.

When globin was administered for several successive days the level of blood globin was measured twelve to eighteen hours after completion of each

TABLE II. ADDIS COUNT (PER 24 HOURS)

DIAGNOSIS		THERAPY	PROTEIN (GM.)	RED CELLS (MILLION/ DAY)	WHITE CELLS (MILLION/ DAY)	CASTS (THOUSAND /DAY)	URINARY SYMPTOMS
Subacute glomerulo-nephritis	1	0	–	15.4	10.1	577	
	2	Globin	1.8	15.8	9.2	1980	Pain in flanks
	3	Globin	4.4	–	–	–	
	4	0	4.7	21.6	12.4	3107	Dysuria
	5	0	2.5	84.0	23.2	20250	
Sprue	1	0	0.160	0.18	7.4	0	
	2	0	0.0	0.22	11.0	0	
	3	Globin	4.7	0.10	13.2	13.3	
	4	Globin	2.1	1.90	7.0	14.7	
	5	Globin	3.2	2.10	8.0	7.0	
	6	Globin	–	–	–	–	0
	7	Globin	3.0	2.00	4.3	1.0	
	8	0	–	–	–	–	
	9	0	0.35	0.55	1.1	0	

infusion. The highest values were observed on the second or third day of treatment. The average value in 24 patients studied was 337 mg., the highest and lowest levels being 1270 mg. and 30 mg. per 100 ml. of blood, respectively. In 18 patients measurement of blood globin was continued after cessation of treatment. In eight instances globin disappeared from the circulation within twenty-four hours and in five was gone by forty-eight hours. In five patients globin was still present forty-eight hours after treatment was stopped; in two of these, globin had disappeared by the fourth day; no further measurements were conducted in the remaining three patients.

Urinary excretion of globin varied considerably and, as noted above, appeared to bear some relationship to the speed of the infusion. In most instances the urinary globin was less than 2.0 Gm. in twenty-four hours, i.e., less than 6 per cent of the administered dose. A few patients excreted larger amounts of globin in the urine; this was especially prominent in those with renal disease but also occurred in patients with apparently normal renal function.

Pleural and ascitic fluid was measured for globin content on seven occasions. In some instances control samples of the fluid were measured for total protein and globin prior to therapy. As shown in Table III, globin does not pass with facility into loculated fluid compartments, concentration in pleural and ascitic fluid being less than that of blood. Globin was still present in ascitic fluid 11 days after cessation of treatment but had disappeared entirely by the thirty-second day.

Changes in Serum Protein.—The results of the determination of serum protein before and after the administration of globin are shown in Tables IV, V, and VI. In Table IV, the 24 cases are divided into two groups, the first containing the cases with a control protein level over 6 Gm. per 100 ml. and the second those patients whose original serum protein value was under 6 Gm. Statistical comparison was made of the values before and after globin therapy, averages and standard deviation being given for the four sets of data. When the control level exceeded 6 Gm., total serum protein was not raised significantly, but in the hypo-

TABLE III. GLOBIN CONTENT OF PLEURAL AND PERITONEAL FLUID

DIAGNOSIS	FLUID	VOLUME (ML.)	TOTAL PROTEIN GM./100 ML.	GLOBIN GM./100 ML.	TIME AFTER GLOBIN RX DISCONTINUED	TOTAL GLOBIN FLUID (GM.)	GLOBIN ADMINISTERED (GM.)	% OF DOSE FOUND
1. (a) Cirrhosis	Ascitic	7600	1.3 (2.2)*	0.058 (0)*	1 day	4.4	128	3.4
(b) Cirrhosis	Ascitic	5000	2.8 (1.6)	0.033 (0)	1 day	1.7	160	1.1
2. (a) Disseminated lupus	Ascitic	1100	–	0.050	12 hours	1.65	72	2.3
(b) Disseminated lupus	Ascitic	4300	4.54	0.220	1 day	9.5	290	3.3
3. Nephrosis	Ascitic	3550	0.760	0.216	1 day	7.70	128	6.0
4. Hodgkin's	Pleural	3200	4.4 (4.0)	0.080 (0)	2 hours	2.6	96	2.7
5. Nephrosis	Ascitic	2800	3.9	0.040 (0)	11 days	1.1	96	1.1
	Ascitic	3500	3.63	0	32 days	0	96	0

*Value in control, pretreatment specimen.

proteinemic patients the increase following globin therapy was highly significant. That this rise in serum protein was not the result of decrease in plasma volume is shown by the fact that the hematocrit values fell from 1 to 4 per cent. Table V shows a similar grouping based upon control serum albumin levels over and under 3 Gm. per 100 ml. of blood. When the serum albumin exceeded 3 Gm. no significant change followed the administration of globin, but in patients with low serum albumin levels, globin therapy resulted in a highly significant rise in the albumin level. Of the hypoalbuminemic patients, three suffered from cirrhosis of the liver, none of whom showed an appreciable rise in serum albumin. If these cases are omitted from Table V, the t value is increased to 3.2, which is significant at the 1 per cent level.

Table VI shows grouping of the same 24 patients based on serum globulin levels over and under 3.5 Gm. Here also there was a statistically significant rise in serum globulin only in those instances of hypoglobulinemia. In some cases there occurred rather striking elevation of serum protein during globin

TABLE IV. TOTAL SERUM PROTEIN AS AFFECTED BY GLOBIN ADMINISTRATION

CONTROL OVER 6 GM./100 ML.		CONTROL UNDER 6 GM./100 ML.	
CONTROL GM./100 ML.	AFTER GLOBIN GM./100 ML.	CONTROL GM./100 ML.	AFTER GLOBIN GM./100 ML.
6.8	7.4	5.4	3.8
8.9	12.0	4.5	10.4
6.0	5.6	4.4	5.8
8.9	8.9	5.5	6.3
9.7	11.4	5.2	6.0
8.4	8.0	4.8	5.2
7.7	8.2	5.2	7.5
7.8	7.9	4.2	5.7
8.6	8.7	5.6	7.5
7.1	7.2	4.4	5.4
6.1	6.2	4.4	5.0
8.0	8.8		
10.6	10.6		
Av. 7.6±1.4	8.5±1.9	4.7±0.6	6.2±1.7
$t = 1.4$ not significant		$t = 2.8$ $p = 1$ highly significant	

TABLE V. SERUM ALBUMIN AS AFFECTED BY GLOBIN ADMINISTRATION

CONTROL OVER 3 GM./100 ML.		CONTROL UNDER 3 GM./100 ML.	
CONTROL GM./100 ML.	AFTER GLOBIN GM./100 ML.	CONTROL GM./100 ML.	AFTER GLOBIN GM./100 ML.
3.9	4.7	1.7	1.8
4.5	4.7	2.1	4.2
3.5	3.8	1.8	2.3
4.7	6.1	2.5	3.1
3.6	3.9	1.9	2.5
3.8	3.9	2.9	3.0
3.6	3.7	3.0	3.0
3.9	4.0	3.0	3.2
3.6	3.7	2.3	2.7
5.1	5.1	2.2	2.6
		2.7	2.7
		2.0	2.2
		2.4	3.0
		1.5	1.6
Av. 4.0±0.4	4.3±0.8	2.2±0.5	2.7±0.6
$t = 1.0$ not significant		$t = 2.5$ $p = 2.0$ highly significant	

therapy, apparently the result of a marked rise in the globulin fraction. Generally the levels returned to normal after three or four days of treatment, but in Table VI it may be noted that the elevation persisted after cessation of therapy in two patients. Inasmuch as this increase was ordinarily of short duration, it could have been due to the presence of large amounts of circulating globin which is precipitated in the globulin fraction by the chemical method used. However, in most instances the rise in globulin greatly exceeded the value for circulating globin. Although measurement of serum polypeptides showed no significant elevation during globin treatment, it is possible that a breakdown product of globin was being measured as globulin by the method employed.

Determination of plasma volume by the dye technique was conducted in three patients with hypoproteinemia. Changes in plasma volume and total circulating protein are shown in Table VII. In a patient with biliary cirrhosis

TABLE VI. SERUM GLOBULINS AS AFFECTED BY GLOBIN ADMINISTRATION

CONTROL OVER 3.5 GM./100 ML.		CONTROL UNDER 3.5 GM./100 ML.	
CONTROL GM./100 ML.	AFTER GLOBIN GM./100 ML.	CONTROL GM./100 ML.	AFTER GLOBIN GM./100 ML.
4.4	7.6	2.9	2.7
6.0	5.9	2.5	2.1
5.0	5.3	1.7	2.0
5.4	5.0	2.4	6.2
3.9	4.3	2.6	3.5
4.2	4.2	1.9	2.4
4.7	4.7	2.7	2.9
4.1	4.0	2.9	2.7
4.4	5.1	2.9	4.8
5.1	5.1	2.0	3.1
3.4	3.5	3.6	5.3
		2.9	2.9
		2.0	2.4
Av. 4.5±0.8	5.0±1.0	2.4±0.5	3.3±1.3
$t = 1.3$ not significant		$t = 3.7$ $p = 1$ highly significant	

TABLE VII. CHANGE IN TOTAL CIRCULATING PROTEIN

DIAGNOSIS	GLOBIN ADMINISTERED (GM.)	TIME	T-1824 SPACE (ML.)	PATIENT WT. (LBS.)	TOTAL PROTEIN (GM./100 ML.)	ALBUMIN (GM./100 ML.)	GLOBULIN (GM./100 ML.)	TOTAL CIRCULATING PROTEIN (GM.)	TOTAL CIRCULATING ALBUMIN (GM.)	TOTAL CIRCULATING GLOBULIN (GM.)
Biliary cirrhosis	–	Control	3700	138	6.2	2.6	3.6	229	96	133
	160	Post-Rx	4075	143	6.2	2.7	3.5	252	110	142
								+23	+14	+ 9
Idiopathic sprue	–	Control	3450	110	4.4	2.4	2.0	152	83	69
	80	During Rx	3400	–	5.3	2.9	2.4	181	99	82
								195	109	86
	176	Post-Rx	3600	116	5.4	3.0	2.4	+43	+26	+17
Idiopathic sprue	–	Control	1975	118	4.4	1.5	2.9	87	30	57
	120	Post-Rx	1850	113	5.0	1.6	3.4	93	30	63
								+6	0	+6

the rise in plasma volume was sufficient to indicate an increase of 23 Gm. in total circulating protein, even though the plasma protein levels were unchanged. In a woman with sprue, marked increase in both protein concentration and plasma volume indicated a rise in total circulating protein of 29 Gm. following the infusion of 80 Gm. of globin and 43 Gm. after 176 Gm. of globin has been administered. In the third patient, also suffering from sprue, the administration of globin resulted in diuresis, loss of peripheral edema, and contraction of the dye space. However, the plasma protein concentration showed minimal increase and thus there was no significant elevation in total circulating protein.

DISCUSSION

In these studies no severe toxic reactions were encountered, and even the mild type of reaction was less frequent with the use of purer lots of globin. The changes in urinary sediment, as determined by Addis counts, are difficult to evaluate since there are minimal data regarding the effect of other protein solutions on the urinary sediment. Brandt and his co-workers noted no untoward effect of globin on renal function either in normal individuals or in patients with cirrhosis of the liver.[8, 9] However, in view of the noted change in the Addis count, it would appear best to avoid the use of globin in patients with renal disease, especially in those suffering from acute nephritis.

Indication from earlier studies that globin disappears rapidly from the circulation has been strengthened by the studies of Berson and his associates[10] who used globin tagged with radioiodine. In these studies, and those of others, much larger amounts of globin were excreted in the urine than was noted in our patients. Most probably the speed of globin infusion plays a part in renal excretion, for in nearly all of our patients globin was administered very slowly. It would appear that globin does not pass with facility into loculated fluid compartments, and even the material which does pass into ascitic fluid can re-enter the protein metabolic pool. Whether globin is broken down into its constituent amino acids with great rapidity, or whether it immediately undergoes only partial degradation is not apparent from our studies. In view of the rapid loss from the circulation, and the absence of marked or

sustained rise in urinary nonprotein nitrogen, it would appear that a substantial portion of the infused material can be used for protein synthesis. This is borne out by the rather striking rise in serum protein noted in the hypoproteinemic patients. In his original studies, Strumia found a greater elevation of total circulating protein than would be expected from the amount of globin administered, and this appears to be true in the cases reported here.[11] It is obvious that the small number of plasma volume determinations permit no conclusions regarding the quantitative rise in circulating protein, but the rise in serum protein concentration cannot be explained on the basis of contraction of the plasma volume. Although it is possible that globin could act in a fashion similar to hypertonic saline, by bringing protein into the plasma compartment, one would expect a greater rise in normoproteinemic patients than in the individuals with low serum albumin.[12]

The fact that no rise in serum albumin concentration occurred in three instances of cirrhosis of the liver, despite the marked hypoalbuminemia in each case, would suggest that the liver is instrumental in synthesis of plasma protein from globin. However, one of these patients, in whom such measurement was conducted, showed a moderate rise in total circulating protein. In addition, there was no greater increase in blood levels of globin nor more prolonged urinary excretion of that substance in patients with hepatic disease than was observed in other individuals. Thus, although it appears probable that globin is rapidly utilized for protein synthesis, it is possible that a substantial portion is broken down into small moieties which pass into interstitial fluid and thence undergo slower, complete degradation.

SUMMARY

The administration of 355 units of globin to 40 patients resulted in no more than mild toxic reactions in less than 8 per cent. In two of three cases studied, significant change in the Addis count was noted during globin administration.

Modified human globin appears to leave the circulation with marked rapidity, although measurement of ascitic and pleural fluid demonstrated only small amounts of globin to be present.

The most striking change following the administration of globin is a substantial rise in serum protein concentration noted only in hypoproteinemic patients. No significant change in serum protein occurs in the normoproteinemic individual. In three patients with cirrhosis of the liver no rise in serum protein concentration accompanied the infusion of globin, despite the low control level of albumin. In one of these instances, however, measurement of the dye space indicated a moderate increase in total circulating protein.

These studies indicate that globin is a satisfactory form of parenteral protein alimentation.

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LABORATORY METHODS

A VIBRATORY MICROTOME FOR SECTIONING LIVING TISSUE

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MADISON, WIS.

RECENT advances in microscopy demand mictrotome techniques which will permit cutting thin uniform sections of living tissue. The elasticity of living tissue renders sectioning difficult and presents a problem which cannot be overcome by increased sharpness of the knife. Hardening the tissue by freezing solves the problem only in investigations for which distortions by ice crystals are unimportant.

The cutting ability of a knife can be improved by imparting to it a rapid slicing movement in the plane of the section. This is the principle of free-hand sectioning where the action of the knife is similar to that of a saw. An application of this sawing principle appears in the use of a high-speed rotating disk-cutter as a microtome knife reported by Ladd (1949). However, the high-speed rotating knife is useful only in cutting dry material. For histologic purposes, and particularly for cutting living tissue, this type of microtome cannot be used because the rotating knife has an intense drying action.

Krause (1940) reported that the cutting ability of the knife can be improved by imparting to it ultrasonic vibrations in the plane of the section. However, the use of ultrasonic vibrations is not practical because of a strong heat effect and vibrations appearing in planes other than the cutting one. Furthermore, the small amplitude of vibration (from $\frac{1}{100}$ to $\frac{1}{1000}$ mm.) does not contribute greatly to the cutting ability of the knife.

The vibratory microtome described herein has a knife which vibrates at a low frequency (100 to 300 c.p.s.) with an amplitude of 0.8 to 0.1 mm. The vibrations are imparted to the knife blade along its longitudinal axis in the plane of the section, and the result is similar to the sawing effect of the high-speed rotating disk cutter. The vibrations increase the cutting ability of the knife to such extent that elastic living tissues can be cut very thin, almost as if they were rigid. Because of lower amplitude the higher frequencies tested, from 300 to 1000 c.p.s., proved less satisfactory.

In the first model, designed by the author and built in 1949 at the R. Jung Co., Heidelberg, Germany, the vibration of the knife was produced by two

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Received for publication, Sept. 17, 1952.
*Research assistant.

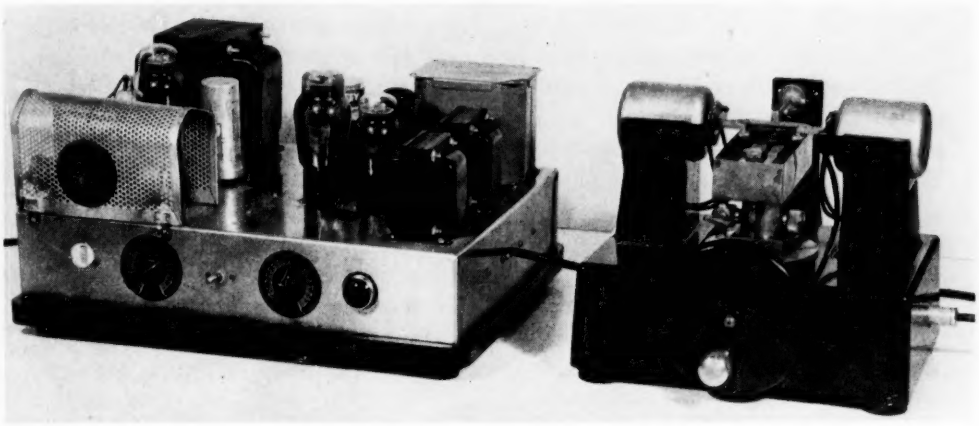

Fig. 1.—The vibratory microtome.

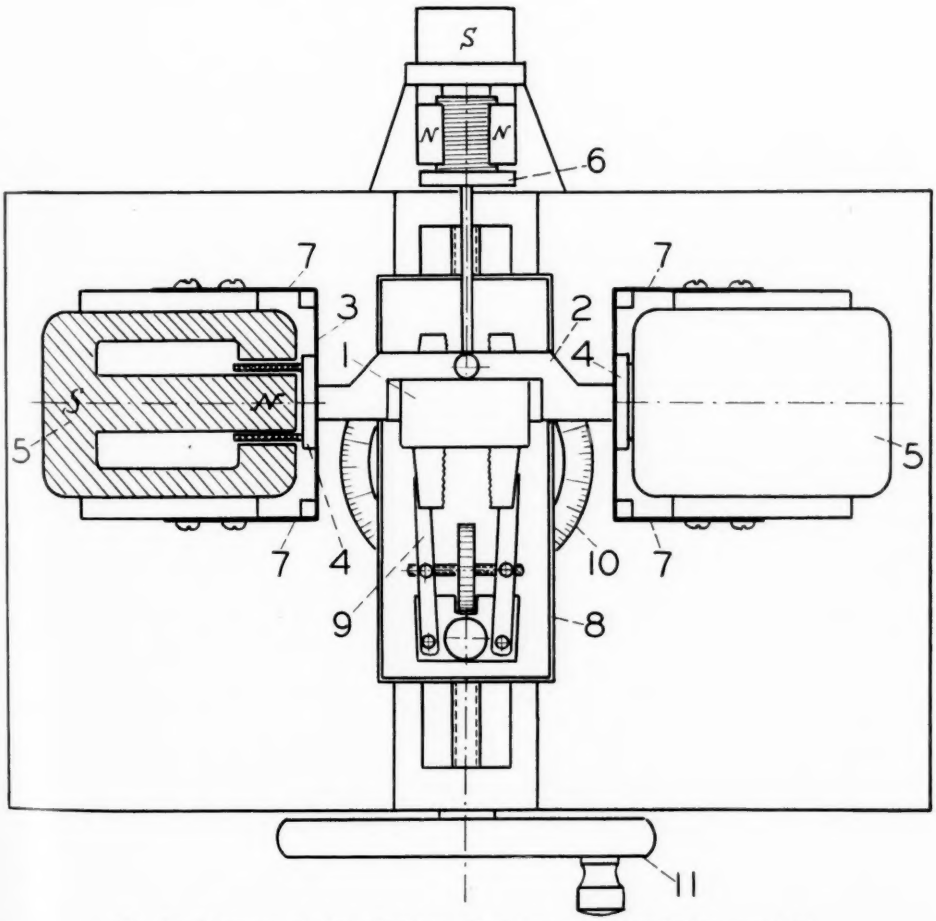

Fig. 2.—Diagram of the vibratory microtome; for description, see text.

electromagnets to which direct current was applied alternately by a make-and-break circuit. The specimen, held in a specially constructed holder, was sectioned under water. Good sections of living animal and plant tissues, varying in thickness from 8 to 30 μ (depending upon the consistency of the material), were obtained in tests at the Institutes of Human Anatomy and of Botany at the University of Heidelberg.

The present model, Figs. 1 and 2, was constructed in 1952 at the University of Wisconsin. The vibration of the knife in this model is produced by means of an A.C. electrodynamic system as in a loudspeaker. A specially made oscillator, with a frequency range of 60 to 1000 c.p.s., serves as a power source. The knife, *1* in Fig. 2, employed in this model is a Rolls-razor or else an Injector-razor blade used with a special interchangeable holder, *2*, which is

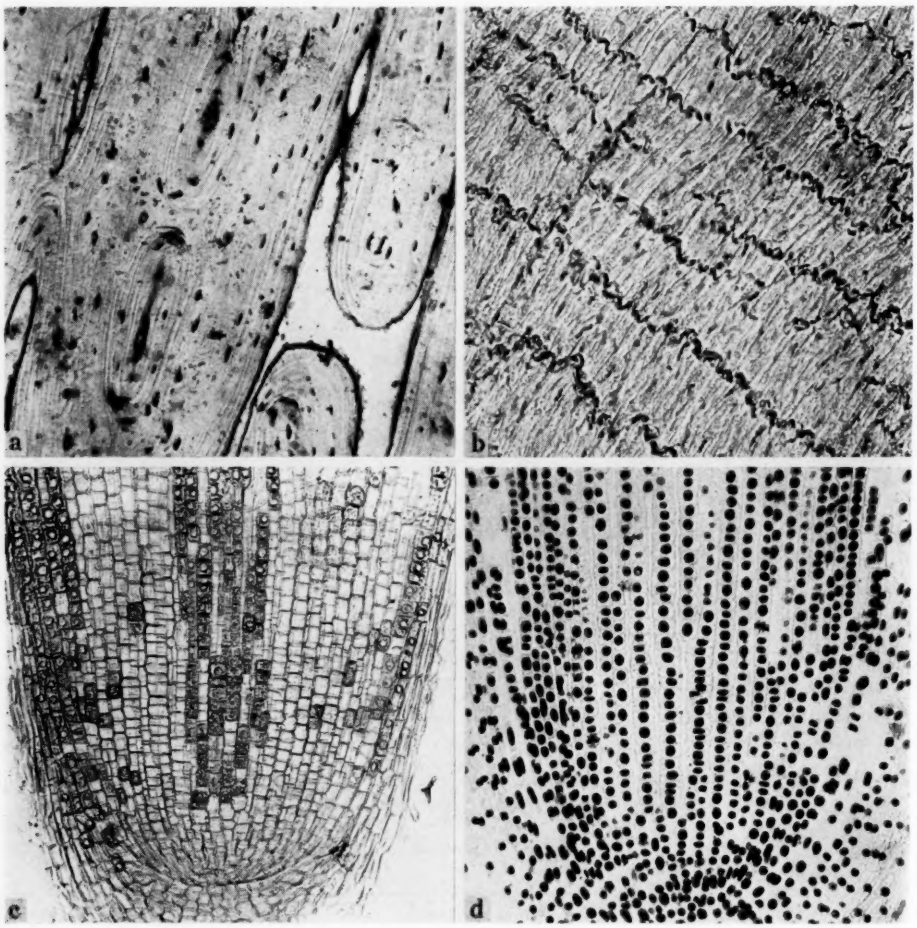

Fig. 3.—Diverse tissues sectioned with the new vibratory microtome. *a*, Longitudinal section of fresh cow femur, 5 μ thick (Giemsa stain). *b*, Fresh chicken crop (smooth muscle) 5 μ thick (Giemsa stain). *c*, Longitudinal section of fresh onion root, 10 μ thick (unstained). Contents remain only in intact cells. *d*, Longitudinal section of fixed, but not imbedded, onion root, 10 μ thick (Feulgen stain).

fastened between two leaf springs, *3*. Each leaf spring is attached on one side to an end of the knife holder and on the other side to a movable coil, *4*, with fixed permanent magnets, *5*, in each coil. Whereas the first models gave only reciprocating motion of the knife, the latest model also has a circular or elliptic motion. This is made possible by a special elastic suspension of the knife and is the resultant of two motions directed at right angles to each other. One is a reciprocating longitudinal motion in the plane of the section, depending on two coils, *4*, *4*. The other is a transverse motion in this plane and is caused by a third coil, *6*, which operates with a 90 degree displacement in phase of current with respect to that of the other two coils. This third coil is attached to the back of the knife holder. A pair of parallel leaf springs, *7*, attached at right angles to each of the previously mentioned springs, permits transverse vibration of the knife. The circular or elliptic motion insures that the cutting edge of the knife presses against the material only when moving sideways. Satisfactory frequencies vary from about 100 to 300 c.p.s., the most favorable depending upon such factors as cell size and elasticity. Between the magnets there is a water tank, *8*, with the specimen holder, *9*, in it. Thus the knife is immersed in water (or in a solution isotonic with the tissue) and sections are cut beneath the water level. A micrometer screw, *10*, at the base of the specimen holder permits adjustment of the thickness of the sections. A hand crank, *11*, moves the specimen holder with respect to the knife. Small pieces of material are held between two pieces of elder pith or cork in the specimen holder.

This model is capable of cutting sections as thin as 3 μ. The smoothness and general quality of the sections approach those obtained from embedded or frozen material. Examples of different tissues sectioned with this microtome are illustrated in Fig. 3.

Since this vibratory microtome cuts thin uniform sections of fresh tissue, it is an effective new tool in certain histologic and cytologic techniques. The sections can either be used for immediate observation or fixed. Their thinness enables penetration of processing fluids to be both rapid and even. Recently they have been used for microphotometric determinations of DNA and in estimating the mitotic index, as will be described in forthcoming publications from this laboratory.

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A RAPID METHOD OF RHESUS GROUPING USING AN AUTOMATIC SHAKER

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VARIOUS short methods of Rhesus grouping, each with their respective advantages and disadvantages, have been described.

The following method has been found to give accurate and clear results.

MATERIALS AND METHODS

Apparatus.—(a) Automatic (Kahn) shaker and tube rack. (b) Rimless tubes 7.5 by 1 cm. and 50 by 7 mm. (precipitin tubes). (c) Pasteur pipettes with rubber teat and graduated to deliver 0.01 ml. (d) Glass slides. (e) Grease pencil.

Performance of Test.—Whole blood taken into Wintrobe's double oxalate is used. 0.01 ml. of anti-D serum is delivered to the bottom of a precipitin tube and 0.01 ml. of blood added. This tube with its contents is put into a 7.5 by 1 cm. rimless tube which is then inserted into a rack in the automatic shaker, set at 275 oscillations per minute. The tubes are shaken for four minutes after which they are removed from the rack. The precipitin tube is then removed from the outer tube; 0.01 ml. of saline is added and the contents are gently but well mixed, after which they are removed with a Pasteur pipette and placed on a slide inside a small circular area previously marked out with a grease pencil. The slide is rocked for three to four minutes and the result read macroscopically and microscopically. No warming is required.

RESULTS

Two hundred tests were performed in parallel with the two-hour tube method and with Diamond's slide method (Diamond and Abelson, 1945) using 0.01 ml. of anti-D serum and an equal quantity of cell suspension or whole blood, respectively.

The results are shown in Table I.

No false results were obtained with either Diamond's slide method or the method described.

TABLE I

METHOD	NO. OF TESTS	POSITIVE	NEGATIVE	FALSE POSITIVE	FALSE NEGATIVE
Tube	200	164	36		
Diamond	200	164	36	0	0
Shaker	200	164	36	0	0

DISCUSSION

The most satisfactory of the rapid methods of Rhesus grouping would appear to be the tube centrifuging method. Using their own technique for this method Poole and Williams (1951) obtained no false results in 540 tests. This method unfortunately takes twenty-five to thirty-five minutes to perform which detracts greatly from its value as an emergency procedure.

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The Chown capillary tube method gives an error of about 2.3 per cent (Discombe and Meyer, 1948; Poole and Williams, 1951). On the other hand, Chown and Lewis (1951) state that "There will be no false positives if the directions are followed," and later "If one is using a good serum real false negatives never occur." It seems that the Chown method is too inaccurate for routine emergency Rhesus grouping except in the hands of those who have much experience of it.

The Diamond slide test is the most convenient of the rapid methods of Rhesus grouping. Provided that a suitable anti-D serum is used, the only danger of this method is a false positive as the result of heavy rouleaux formation (overheating should not occur if the test is performed properly). Rouleaux formation can be differentiated by microscopic reading and dispersed by adding a drop of saline after the slide has been heated. In my experience rouleaux formation can always be differentiated from agglutination by these means. This method is, however, often condemned because of the possible confusion that may arise in mistaking rouleaux formation for agglutination.

The shaking method, described above, is simple to perform and does not require great technical experience. Rouleaux formation does not occur, and the results are easy to read. It is essential to read the test both macroscopically and microscopically. It is suggested that this method is conjunction with the Diamond slide test will provide an accurate and reliable joint method of emergency Rhesus grouping.

SUMMARY

A rapid method of Rhesus grouping is described and the results of two hundred tests are given. The rapid methods of Rhesus grouping are discussed. It is suggested that this test in conjunction with Diamond's slide method will provide an accurate and reliable method of emergency Rhesus grouping.

I would like to express my thanks to Dr. Frank Kellett for his kind help and suggestions during the preparation of this paper.

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RAPID DETERMINATION OF BLOOD ACETONE AND BLOOD GLUCOSE

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NEW ORLEANS, LA.

INTRODUCTION

THERE are many occasions when the values of blood sugar and blood acetone are of extreme importance in both diagnosis and management of varied medical diseases. Several excellent tests for the determination of blood glucose and acetone are now available. They are, by and large, restricted in their scope in that the equipment required is usually found only in large laboratories or hospitals, but rarely in the laboratory of a practicing physician. They are further handicapped by their time-consuming nature; this is especially serious, since rapid determinations are often required in periods of medical emergency, such as coma and shock.

In reviewing the literature[1, 7, 8, 9, 10, 11] for tests which would obviate these defects one finds that those which require less equipment require more time and vice versa. However, Kleeberg,[3] in his use of the old Moore's test,[4, 6] has, in the opinion of this observer, perfected a simple, practical, qualitative means of determining the blood sugar level. This paper presents a modification of Kleeberg's test whereby both the blood sugar and the blood acetone level are obtained with a minimum of equipment and time.

MATERIALS AND METHOD

The materials required for this test can easily be carried in the doctor's bag and include the following:

(a) 4 oz. bottle of 20 per cent trichloroacetic acid.
(b) 2 oz. bottle of 20 per cent sodium hydroxide.
(c) 2 oz. bottle of aqua ammonia (strong).
(d) 1 oz. bottle of 20 per cent sodium nitroprusside.
(e) 2 test tubes, preferably with screw caps.
(f) Funnel, small, and filter paper.
(g) Hypodermic syringe, 5 ml.

These amounts, of course, may be varied, but they represent sufficient material for 30 tests. The test apparently works equally well with 20 per cent potassium hydroxide,[3] and this solution may be used if 20 per cent sodium hydroxide is not available.

For convenience sake the test tubes are graduated, avoiding the need for pipettes. The first tube is marked at 4.0 ml. and 8.0 ml. levels, while the second is marked at 1.5 ml. and 4.5 ml.

The test is done as follows (see Fig. 1): four centimeters of the patient's blood are added to 4 ml. of the 20 per cent trichloroacetic acid. The tube is capped and shaken vigorously for thorough mixing. Then, if a centrifuge is available, it is centrifuged for one

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minute. If a centrifuge is not available, then the solution may be filtered. The supernatant fluid of filtrate is then divided, 3 ml. being used for the glucose determination and the rest for the acetone level. The 3 ml. is added to the second tube with 1.5 ml. of 20 per cent sodium hydroxide. The tube is then heated with a glass bead for approximately one minute or to boiling. If several tests are being done at the same time, possibly in a hospital, the tubes may be heated simultaneously in a water bath for five minutes. The resulting color change of colorless through the stages of yellow to orange is then read as follows:

(a) Colorless to tinge of yellow—hypoglycemia	0
(b) Pale or lemon yellow—normal	1
(c) Deep yellow—hyperglycemia	2
(d) Orange—severe hyperglycemia	3

These readings can be made with daylight or artificial light; however, fluorescent or white light is best for reading against standards.

Standards can be made by applying this same test to stock glucose solutions, using 3 ml. of stock solution and adding 2 drops of trichloroacetic acid and heating. The standard will retain its color for twelve to twenty-four hours.

To obtain the acetone level, 1.5 ml. or the remainder of the filtrate is used. Add 2 drops of 20 per cent sodium nitroprusside and overlay with aqua ammoniae. If a purple ring forms between the filtrate and ammonia the test is positive. The intensity of the ring signifies the quantity of acetone.

RESULTS

We have used the test frequently in the admitting room and on the wards at Charity Hospital in New Orleans with gratifying results.

CASE 1.—A 32-year-old Negro man, a known diabetic patient for eight years, on 70 units NPH, was seen in a coma in the admittance room at 7:00 A.M. His father had been unable to arouse him and had therefore brought him to the hospital. A catheterized urine specimen proved to contain 3+ sugar and a trace of acetone. At the same time the rapid blood sugar and acetone test was applied to 4 ml. of his blood. This showed a definite hypoglycemia and no acetone. Fifty milliliters of 50 per cent dextrose was given intravenously at once. The patient revived almost immediately and stated that he had taken his insulin the previous morning and then took a nap before lunch. He failed to eat lunch and slept through supper and never did wake up until after receiving the dextrose in the admittance room. Thus the test was confirmed and, incidentally, the danger of relying solely on residual urine was illustrated.

CASE 2.—A 23-year-old Negro woman on the psychiatric service was being given insulin shock therapy. After dextrose was administered to terminate the treatment she failed to respond. It was difficult to ascertain whether she was in a catatonic state, or whether she had not received sufficient dextrose to overcome an insulin sensitivity. The rapid blood glucose test was applied and proved the presence of a hypoglycemic state. Further administration of dextrose brought the patient back to consciousness.

CASE 3.—A 22-year-old white woman in coma was admitted to the ward at 11:00 P.M. The urine was strongly positive for both sugar and acetone. Insulin, fluid, and electrolyte therapy were administered at once. Every hour, in addition to the urine examinations, the rapid sugar and acetone tests were applied. As the patient responded to therapy the tubes were saved and compared, showing a gradual diminution of the intensity of the yellow color as well as a negative acetone. As suggested by Kleeberg, this comparison of tubes is in itself a valuable feature in the diagnosis and prognosis of the patient.

In another case there was no response to therapy, as demonstrated by the tests, and the patient died six hours following admission.

RAPID DETERMINATION OF BLOOD ACETONE AND BLOOD SUGAR LEVELS

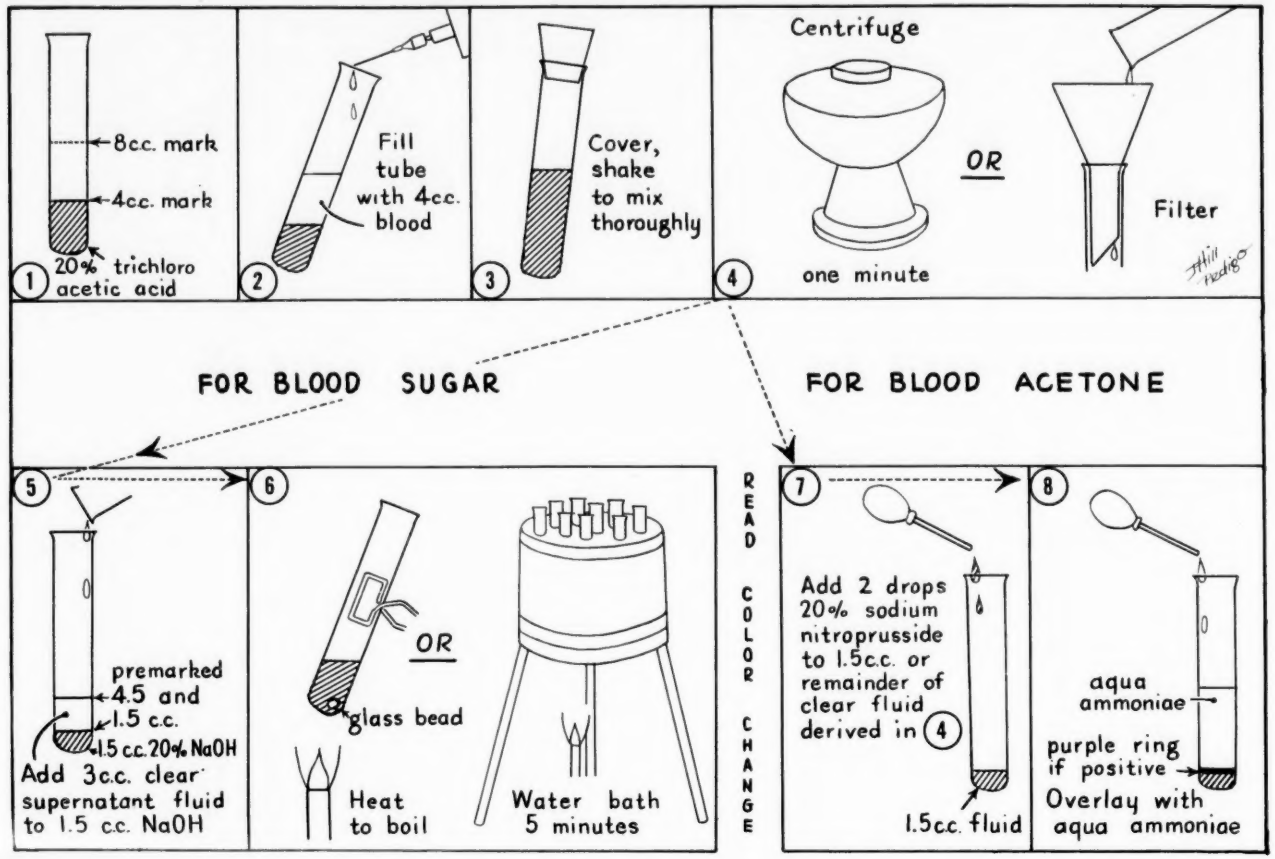

Fig. 1.

DISCUSSION

Although this test is primarily a qualitative one, some quantitative value may be obtained by comparison with stock solutions of different quantities. Therefore we made several different stock solutions such as 50, 75, 100, 125, 175, 200, 250, 300, and 400 mg. per cent and applied the test to these solutions. Kodachromes were taken of the results, and the transparencies were used as standards. Our readings were then compared with the Folin-Wu method,[2] with excellent parallel, as illustrated in Fig. 2.

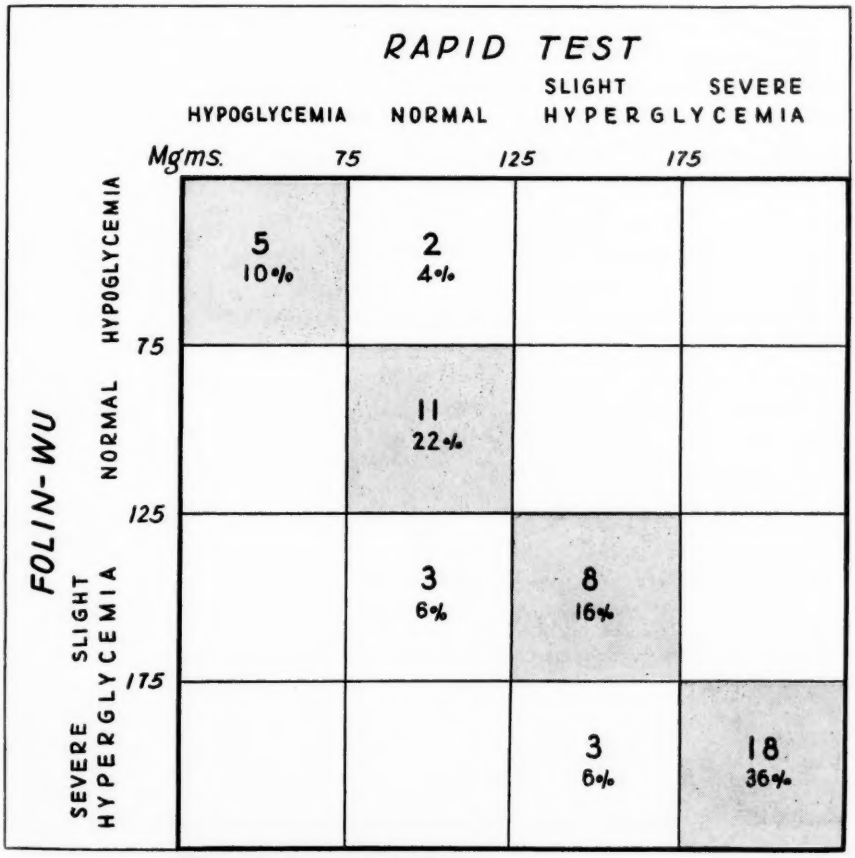

Fig. 2.—Comparison of fifty blood samples (diabetics and nondiabetics) using the Folin-Wu and the rapid determination methods for the determination of blood sugar. Note that 84 per cent of the samples correlate within the same range.

There is a slight error in this test, presumably due to creatine and uric acid which react to the sodium hydroxide, but this is rarely more than a 20 mg. per cent difference and varies so much from patient to patient that we do not correct for it. It must be remembered that this is not intended to be a sensitive test of finite quantities. After all, if a patient is in coma we want

to know if his blood sugar is high, be it 250 mg. or 270 mg.; and on the other end of the scale, a diabetic patient in coma with a reading of 50 mg. or 70 mg. needs glucose immediately. Jaundice, uremia, and "yellowing" drugs such as atabrine do not effect this test, as the trichloroacetic acid produces a protein free, clear, colorless filtrate.[5] Actually this is a clinical test intended to provide the average physician with an additional diagnostic tool, not to replace another test.

The acetone test is basically a modification of Legal's test for urine acetone[12] once the protein-free filtrate is obtained. It is simpler, however, because no acidification with glacial acetic acid is necessary. It also reveals a truer clinical picture of the patient's status by avoiding the errors sometimes produced by residual urine.

SUMMARY AND CONCLUSIONS

The need for a simple test for blood sugar and acetone levels, which does not require elaborate equipment and is not time consuming, is stressed.

A method of obtaining these levels with virtual "bedside" technique, based on modification of Kleeberg-Moore's test and Legal's acetone test, is presented.

While this test is not meant to be a sensitive quantitative test, or to replace the standard determinations of this sort, it is offered as a valuable means of obtaining information which ordinarily would not be available due to lack of equipment or necessity for speed.

The results of several cases are listed, as well as comparison of results with the standard Folin-Wu method.

I wish to offer my sincere thanks to George Pratt and to my wife, Beatrice Yager, for their technical assistance.

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A SPECTROPHOTOMETRIC DETERMINATION OF DIOXYLINE IN PLASMA

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DIOXYLINE is a synthetic isoquinoline derivative similar in structure to papaverine. The chemical name is 6,7-dimethoxy-1-(4′-ethoxy-3′-methoxybenzyl)-3-methylisoquinoline, and shall be referred to as Dioxyline in the remainder of this paper. Pharmacological studies[1] and clinical observations[2, 3] concerning Dioxyline have been published.

The need for determining the blood level concentrations of Dioxyline in current clinical and pharmacologic studies made it necessary to develop an adequate chemical method for the determination of this drug in the blood.

This paper describes a procedure for the extraction of Dioxyline phosphate or Dioxyline tartrate in the form of the free base from human and dog plasma with a subsequent ultraviolet spectrophotometric determination of the Dioxyline in dilute hydrochloric acid at 252 mμ. This method has been used for the determination of Dioxyline in a study of the destruction of Dioxyline in dogs,[4] and it is the method used by this laboratory for the routine assays of Dioxyline in human plasma samples submitted by various clinicians.

EXPERIMENTAL

A study was made first of the relationship between concentration of Dioxyline and absorbency at the maximum band (252 mμ) in the following manner. The absorbencies of Dioxyline tartrate solutions varying in concentrations from 0.25 to 7.5 micrograms per milliliter in 0.5N hydrochloric acid were measured in 1 cm. silica cells using a Beckman Model D.U. spectrophotometer with a slit width adjustment of 1.1 mm. The results of this experiment, illustrated in Fig. 1, show the conformity to the Beer-Lambert law for solutions within the concentration range indicated.

The ability to recover Dioxyline which had been added to dog or human citrated plasma was studied in the following experiment. Citrated human plasma was prepared by adding 10 ml. of a 10 per cent sodium citrate solution to 100 ml. of sterile, nonpyrogenic human plasma containing no anticoagulant. Citrated dog plasma was prepared in the same manner. Separate 1 ml. portions of aqueous solutions of Dioxyline tartrate containing from 12.5 to 500 micrograms per milliliter were diluted to 25 milliliters with the citrated human plasma to yield final concentrations ranging from 0.5 to 20.0 micrograms of Dioxyline tartrate per milliliter of final solution. Adequate dilutions were made with the citrated dog plasma in the same manner as with human plasma to yield final concentrations ranging from 1.0 to 5.0 micrograms of Dioxyline tartrate per milliliter of final solution. These plasma solutions containing the drug were subjected to the procedure to be described for the determination of Dioxyline in plasma.

The recovery data from these determinations performed on the prepared known dilutions are shown in Table I. It appears that the optimum concentration range yielding the greatest accuracy in the analysis is between 1 and 5 micrograms of Dioxyline tartrate

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per milliliter of plasma. A one microgram concentration in the plasma is equivalent to a concentration of 0.20 microgram per milliliter in the final acid solution. Below this concentration the difference in the absorbency of the blank and the total absorbency becomes so small that the accuracy diminishes rapidly.

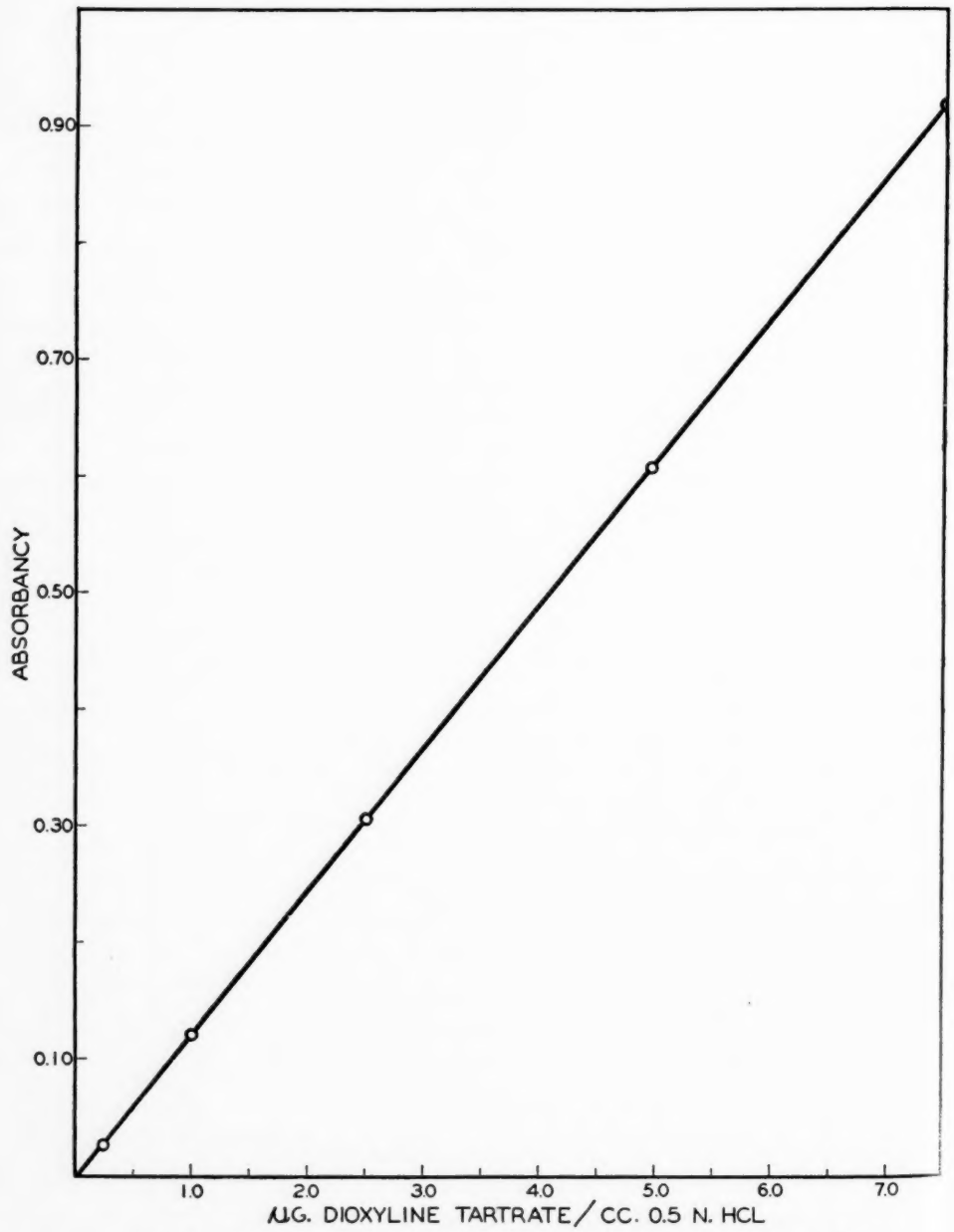

Fig. 1.—Calibration Curve. Solutions of Dioxyline Tartrate in 0.5N. HCl.

Due to the rather low solubility of Dioxyline base in ether the extraction appears to be somewhat incomplete for plasma solutions containing 20.0 or more micrograms of Dioxyline tartrate per milliliter.

Spectra of the acid solutions obtained from a human citrated plasma blank and from citrated plasma containing 15 micrograms of Dioxyline tartrate per milliliter (equivalent to 3.0 micrograms per milliliter in the final acid solution) are compared with the spectrum of a solution containing 3 micrograms of Dioxyline tartrate per milliliter in 0.5N hydrochloric acid in Fig. 2. This experiment shows that the extraneous absorbing materials extracted from the citrated plasma do not exhibit a marked interference at 252 mμ. Therefore, the absorbing portion of the Dioxyline molecule is not appreciably affected by the extractable components of the citrated plasma at this wave length. It was also found that plasma blanks from human blood containing heparin as the anticoagulant exhibited no greater absorption at 252 mμ than did citrated plasma blanks.

Human plasma was obtained from a patient before and two hours after the oral administration of a nine grain tablet of Dioxyline Phosphate. The spectra of the acid ex-

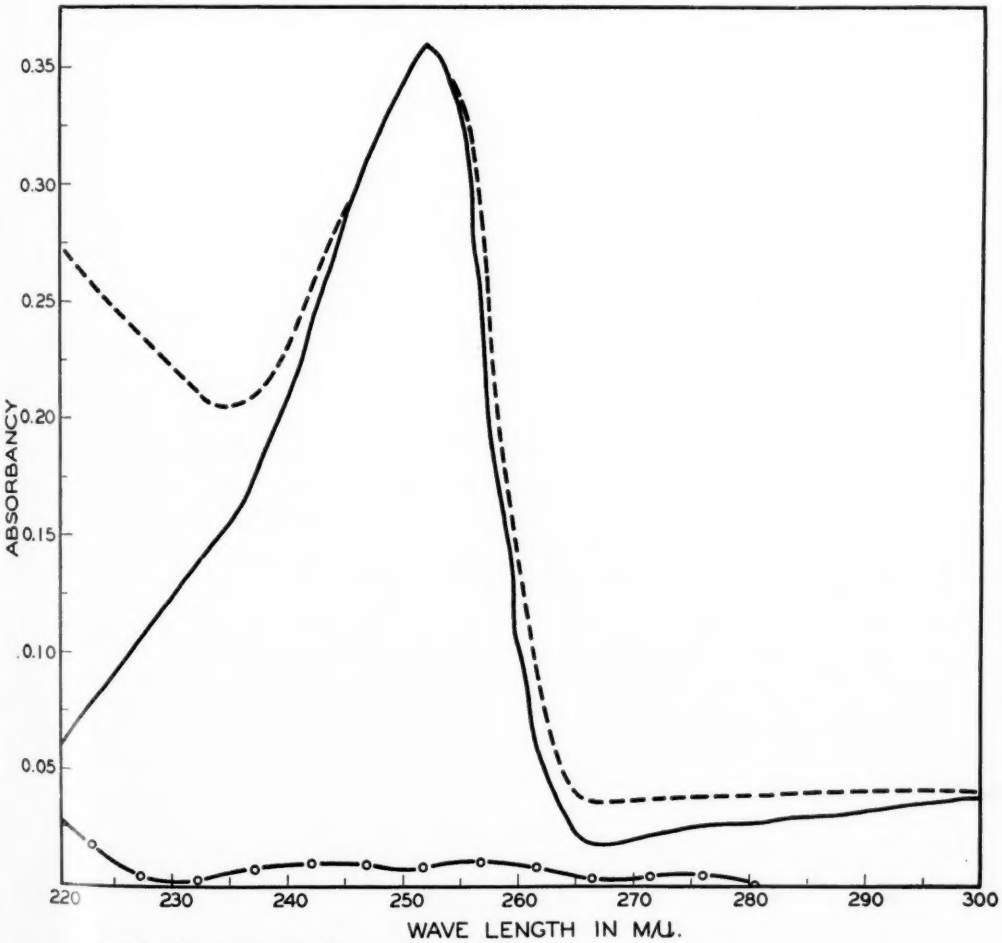

Fig. 2.—Absorption Spectra.
— 0 — Extract from human plasma blank.
——— Solution of Dioxyline Tartrate in 0.5N. HCl (3.0 μg/c.c.).
– – – Extract from citrated human plasma containing 15 μg Dioxyline Tartrate per c.c. (final acid solution, 3.0 μg/c.c.). Cary Recording Spectrophotometer.

tract from the plasma blank and the plasma containing Dioxyline are compared in Fig. 3. A comparison of the spectrum of the acid extract with that of Dioxyline shown in Fig. 2 indicates that the absorbing portion of the material extracted from the plasma of this patient is the same as that for Dioxyline.

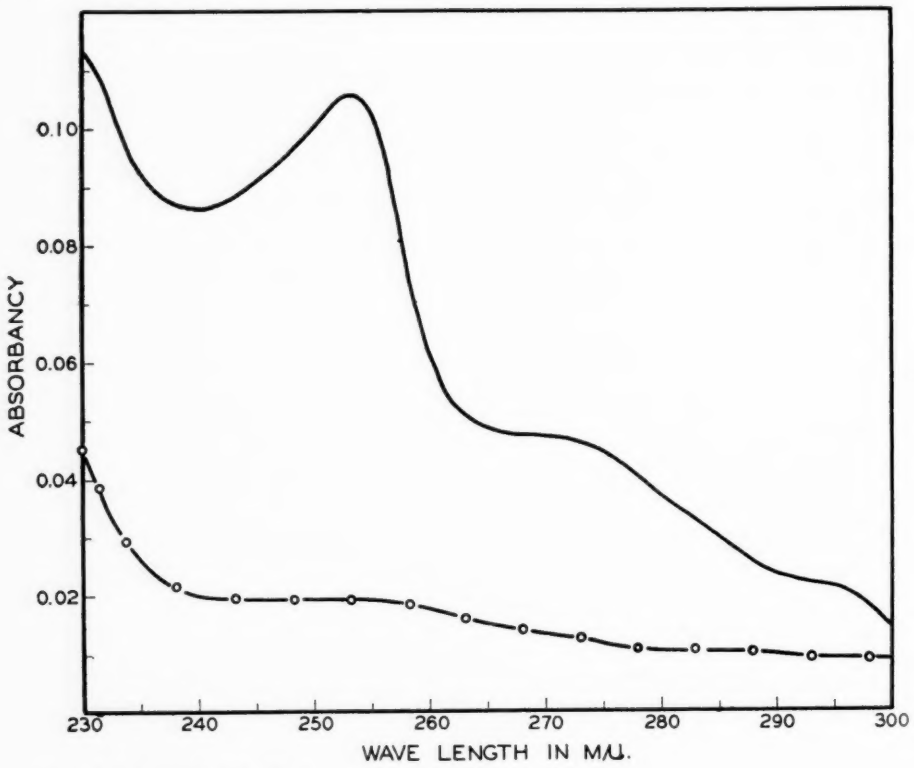

Fig. 3.—Absorption Spectra.
— 0 — Extract from human plasma blank.
——— Extract from human plasma obtained from same patient following oral Dioxyline Phosphate administration. Cary Recording Spectrophotometer.

The above experiment was repeated with citrated dog plasma obtained twenty minutes after the injection af 20 mg. per kilogram of Dioxyline tartrate intravenously. The results of this experiment were essentially the same as those obtained from human plasma.

The dog plasma obtained from the above experiment was stored in a refrigerator for eight days and assayed according to the procedure described below. After two hours it was found to contain 11.8 micrograms per milliliter, after one day—12.0 micrograms, after three days—12.0 micrograms, and after eight days—12.6 micrograms. Thus it appears that plasma-containing Dioxyline may be stored at refrigeration temperature for at least three days with no appreciable change in the amount of drug extracted.

METHOD

Apparatus.—(a) Extraction tubes—A one millimeter micro stopcock was sealed onto a 40 milliliter glass-stoppered centrifuge tube. (b) Filtering funnels—The stem of a 40 millimeter funnel was elongated sufficiently to extend into the bulb of a 25 milliliter volumetric flask. (c) Spectrophotometer—A Beckman Quartz Model D.U. spectrophotometer and 1 cm. matched silica cells were used.

TABLE I. RECOVERY DIOXYLINE TARTRATE FROM PLASMA

MICROGRAMS DIOXYLINE TARTRATE/ML. PLASMA ADDED	MICROGRAMS DIOXYLINE TARTRATE/ML. PLASMA FOUND	
	HUMAN	DOG
0.5	0.7	–
0.5	0.7	–
0.5	0.7	–
0.5	0.7	–
1.0	1.0	1.0
1.0	1.1	1.0
1.0	1.0	1.0
1.0	1.0	1.0
3.0	–	2.9
3.0	–	3.1
3.0	–	2.9
3.0	–	3.0
5.0	5.1	4.9
5.0	4.9	5.1
5.0	4.9	5.0
5.0	5.0	5.0
10.0	10.0	
10.0	10.2	
10.0	10.2	
10.0	10.0	
15.0	14.9	
15.0	14.7	
15.0	14.7	
15.0	14.7	
20.0	19.4	
20.0	19.4	
20.0	19.5	
20.0	19.4	

Table I.—Recovery Data: Results obtained from four determinations on different concentrations of Dioxyline Tartrate in citrated plasma.

PROCEDURE

Centrifuge the blood samples and obtain the clear plasma solution. Transfer exactly 5 milliliters of the plasma into the extraction tube and add 2 milliliters of ammonia test solution (U.S.P.). Extract with three 10 milliliter portions of ether and collect the ether phases in another extraction tube. Add three drops of distilled water to the combined ether phases, and without shaking discard the aqueous portion. Extract the combined ether extracts with three 5 milliliter portions of 0.5N hydrochloric acid. Filter each acid extract through Whatman No. 42 paper (previously washed with water and 0.5N hydrochloric acid) into a 25 milliliter volumetric flask. Wash the paper with 5 milliliters of 0.5N hydrochloric acid and adjust the volume to 25 milliliters with 0.5N hydrochloric acid. Mix thoroughly and measure the absorbency at 252 μ in one centimeter cells using 0.5N hydrochloric acid as the reference solution. Obtain a plasma blank using the same procedure with 5 milliliters of plasma obtained from the same patient before Dioxyline administration.

CALCULATIONS

Micrograms of Dioxyline Phosphate per milliliter of plasma =

$$\frac{A_{252}\ m\mu \text{ observed} - A_{252}\ m\mu \text{ blank}}{0.490^*} \times \frac{4}{1} \times \frac{25}{5}$$

Micrograms of Dioxyline Tartrate per milliliter of plasma =

$$\frac{A_{252}\ m\mu \text{ observed} - A_{252}\ m\mu \text{ blank}}{0.606^\dagger} \times \frac{5}{1} \times \frac{25}{5}$$

Micrograms of Dioxyline Base per milliliter of plasma =

$$\frac{A_{252}\ m\mu \text{ observed} - A_{252}\ m\mu \text{ blank}}{0.490^{*}} \times \frac{4}{1} \times \frac{25}{5} \times \frac{367^{\ddagger}}{514}$$

*Absorbency of 0.5N hydrochloric acid solution containing 4.0 micrograms Dioxyline Phosphate per ml.

†Absorbency of 0.5N hydrochloric acid solution containing 5.0 micrograms Dioxyline Tartrate per ml.

‡Factor for converting Dioxyline Phosphate to Dioxyline Base.

SUMMARY

Solutions of Dioxyline phosphate and Dioxyline tartrate in 0.5N hydrochloric acid exhibit a maximum absorption at 252 mμ, and conformity to the Beer-Lambert law is evident within the concentration range of 0.25 to 7.5 micrograms per milliliter.

The absorption band produced by Dioxyline which has been extracted by the procedure described above from dog or human plasma appears to be qualitatively the same as that produced by Dioxyline in 0.5N hydrochloric acid. Little interference is exhibited at 252 mμ due to extraneous material extracted from plasma.

Dioxyline phosphate and Dioxyline tartrate may be satisfactorily determined quantitatively in plasma, obtained from blood of patients under Dioxyline medication, in concentrations of 1 to 15 micrograms per milliliter of plasma by the spectrophotometric procedure described.

Acknowledgment.—We wish to acknowledge the assistance of Dr. Mary Root and Dr. K. G. Kohlstaedt for gratefully furnishing the plasma samples used in this study and also to John Woerner for his assistance in the preparation of the figures.

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CARBON DIOXIDE AND ROUTINE CULTURES IN THE DIAGNOSTIC LABORATORY

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STIMULATION of *Brucella*, the gonococcus, meningococcus, and *Hemophilus influenzae* by carbon dioxide is a well-recognized phenomenon. Less publicized is the beneficial effect of carbon dioxide upon other organisms, especially the gram-positive cocci, in their primary isolation from the human body. The work of Valley and Rettger,[9] showing the improved growth of many species of pathogens in carbon dioxide, has not been generally applied to the diagnostic laboratory. The present study was undertaken to obtain data on the influence of carbon dioxide when used for routine cultures in hospital laboratories.

METHODS

Essentially, the procedure consisted of preparing duplicate blood agar plates from throat swabs, sputum, pus, and other miscellaneous specimens sent to hospital laboratories for culturing. One plate was incubated in air, the other in a candle jar. They were examined the following morning. No subcultures were used since, for most species concerned, carbon dioxide may be necessary for the primary isolation, but is not needed thereafter. No cultures for *M. tuberculosis* were included in the series because previous work[3] had shown that carbon dioxide was not of benefit for the development of the tubercle bacillus, at least on Petragnani's medium.

A total of 869 cultures were examined; 114 gave no growth.

RESULTS

Findings are tabulated in Table I. Most interesting are the figures on pneumococci; almost half would have been missed in the simple aerobic atmosphere. It is remarkable to view one plate with no growth and its carbon dioxide duplicate with hundreds of colonies of pneumococci. If no pneumococci are present on the aerobic plate at sixteen to twenty-four hours, they do not appear at forty-eight hours, indicating that the function of carbon dioxide is not merely to increase the growth rate.

There were 21 isolates of pneumococci which grew on both plates, but better in the candle jar. In many of these the larger colonies made recognition easier and also furnished adequate material for bile solubility testing or the quellung reaction, which was not possible from the aerobic plate. This is of the greatest practical importance since physicians tend to become discouraged with attempts to make bacteriological diagnoses if findings are not reported at twenty-four hours.

From the Bacteriology Laboratories, Wayne Liberal Arts College.
Cooperating Hospitals: Children's Hospital of Michigan, Detroit Receiving, Wayne County General Hospital, and Grace Hospital.
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TABLE I. GROWTH OF THE ORGANISMS UNDER DIFFERENT ATMOSPHERES
(869 cultures made in duplicate)

TOTAL STRAINS ISOLATED		GREW AS WELL IN AIR AS IN CO_2	GROWTH BETTER AEROBIC	GROWTH BETTER CO_2	GROWTH IN CO_2 ONLY
Beta hem. strep.	163	88	7	30	38 (22%)
Pneumococci	118	43	0	21	54 (45%)
Micrococci	213	192	3	10	8 (4%)
Friedlander-like	27	15	1	11	0
H. influenzae	7	1	0	4	2
Meningococcus	1				1
Diphtheroids	96	58	14 (9 of these did not grow in CO_2)	15	9 (9%)
Monilia	32	12	9 (5 of these did not grow in CO_2)	7	4 (12%)
E. Coli	35	29	2	4	0
A. aerogenes	15	12	2	0	1
Ps. aeruginosae	30	27	2	1 (better pigment)	
Misc. enterobacteriaceae	23	19	2	1	1

Of the beta hemolytic streptococci isolated, 22 per cent required carbon dioxide to initiate any growth. Almost an equal number were stimulated to produce larger or more numerous colonies than when incubated aerobically.

It is surprising that such a relatively nonfastidious organism as *Micrococcus pyogenes* might require carbon dioxide for isolation. A total of 18 strains grew better, or only, in carbon dioxide, compared with three strains which grew better aerobically. Statistical analysis of these figures shows that the chance of such disproportion being accidental is only one in 671.

Diphtheroids and monilia were interesting because for each some strains were completely inhibited by the candle jar while others appeared definitely dependent. Species identification was not made for all monilia, but albicans and tropicalis isolates were found in both the carbon dioxide-dependent and carbon dioxide-inhibited groups. The effect of carbon dioxide on fungi has been studied in connection with food spoilage by many, including Tomkins[8] and Empey and Vickery,[2] who found great variations depending on species or strains of fungus. Figures in the present study indicate that for monilia and diphtheroids results are comparable with and without carbon dioxide, some strains being missed in either atmosphere.

Friedlander's bacillus, on the whole, showed significantly greater growth in carbon dioxide. Most beneficial was the tendency of the colonies to be more mucoid, and thus often type from the carbon dioxide plates when no quellung could be demonstrated by use of aerobic colonies. Not all strains culturally and biochemically resembling Friedlander typed with sera 1, 2, 3, and 4, and such isolates possibly were of higher types. Hence the term "Friedlander-like" in the chart.

As anticipated, isolation of *H. influenzae* and the meningococcus was helped by carbon dioxide. For best growth of Hemophilus, however, chocolate agar was necessary as well as carbon dioxide.

Results with enteric bacilli are summarized because no marked stimulation from carbon dioxide was demonstrated. In 30 isolations of *Pseudomonas aeruginosae* one showed much greater pigmentation in carbon dioxide.

DISCUSSION

It will be questioned whether the results here reported will be repeated with all types of blood agar. It was the general impression in this study that the better the medium, the more improvement could be accomplished by carbon dioxide. The medium employed here consisted of dehydrated tryptose blood agar (Difco) enriched with Supplement B without Dextrose* and human blood. Similar findings were obtained in an unreported study previously conducted[3] in which freshly prepared beef infusion agar plus sheep blood composed the medium. Sheep blood was also employed in the laboratory of Nye and Lamb[4] who reported that carbon dioxide was a significant aid in the isolation of pneumococci, beta hemolytic streptococci, alpha hemolytic streptococci, and other organisms. Thus it may be concluded that carbon dioxide would help in a variety of media.

Human blood was used throughout the present series, as no other was available in the quantity necessary. For added enrichment Supplement B without Dextrose* was used with the blood. It was most interesting that the addition of this substance appeared to nullify largely the inferiority of human blood, suggesting that human blood may be deficient in nutriment rather than poor because of antibody content. Comparative studies should be done with and without such supplement in blood plates before the actual degree of improvement can be stated.

It will also be asked whether carbon dioxide gas alone accomplishes the same change as does a candle jar. Again, this can be answered in the affirmative because of the excellent report of Rose,[5] who found that a capneic (carbon dioxide) incubator fosters the growth of Klebsiella, beta hemolytic streptococci, pneumococci, and certain strains of micrococci. In the unpublished study[3] mentioned above, 10 per cent carbon dioxide gas by volume was admitted to jars. Thus, either a candle or a gas tank may be used. Some laboratories now use carbon dioxide incubators commercially available, or "home-made" by admitting the carbon dioxide through a meter into a relatively airtight incubator.

The effect of carbon dioxide upon Klebsiella is similar to its effect upon *Bacillus anthracis,* as recently reported by Chu.[1] He found that if the anthrax bacillus is grown in carbon dioxide on enriched medium an encapsulated form with a mucoid colony results.

It is evident that we are not original in finding carbon dioxide essential for routine diagnostic purposes. In addition to the authors mentioned above, Smith College has long been aware of its importance, as recommended in their *Laboratory Guide In Diagnostic Bacteriology.*[6]

It is obvious that an aerobic blood plate has no justification in a clinical laboratory unless it is a triplicate to ones in carbon dioxide and anaerobiasis.[7]

*Available from Difco Corporation upon request.

SUMMARY

Data are presented showing that carbon dioxide is needed routinely for incubation of blood plates in the diagnostic laboratory. Without the addition of carbon dioxide to the atmosphere a significant percentage of strains fail to develop; these include especially beta hemolytic streptococci, pneumococci, and micrococci. The most carbon dioxide-dependent is the pneumococcus; 45 per cent of strains were isolated only with the aid of carbon dioxide.

Typing of Friedlander's bacillus was improved by growth in carbon dioxide, since frequently more mucoid colonies were produced.

Diphtheroids and monilia showed some strains carbon dioxide-dependent, other strains completely inhibited. No net loss results when a carbon dioxide atmosphere is used instead of unmodified aerobiasis.

The necessary carbon dioxide may be adequately supplied either with a simple candle jar or with a measured volume of the gas. In the present series 869 cultures on blood agar plates were incubated in duplicate, aerobically and in a candle jar.

Supplement B without Dextrose (Difco) proved a useful adjunct to improve the otherwise poor growth typical when human blood is used to enrich culture media.

Appreciation is expressed to Dr. William Hale who first suggested such a study to the senior author, and under whose direction a similar comparative study was made earlier.

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DETERMINATION OF TOTAL PROTEIN IN SMALL QUANTITIES OF SPINAL FLUIDS: CONFIRMATION OF THE ACCURACY OF A COLORIMETRIC METHOD USING FOLIN-CIOCALTEAU REAGENT IN THE PRESENCE OF COPPER

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INTRODUCTION

IN A previous publication[1] we stressed the need of the public health laboratory for a method permitting the estimation of total protein in small quantities of spinal fluid and described an adaptation of the VDRL turbidimetric method[2] designed to fill that need. Continued experience with the modified VDRL method has demonstrated, however, that the accuracy of results is less than that which is desirable whenever a specimen shows a marked departure from the albumin-globulin ratio of normal spinal fluid. In other words, the effect of variation in A/G ratio is much greater on the modified than on the original VDRL method. Consequently, publication[3] of a sufficiently sensitive colorimetric method prompted us to investigate its possibilities since a reliable colorimetric method theoretically would be free from the inherent inaccuracies of turbidimetric methods for determining the sum total of different proteins producing different sizes of particles in the presence of any precipitant.

MATERIALS AND METHODS

Solutions of known, Kjeldahl-controlled concentrations of total protein, ranging from 10 through 140 mg. per 100 ml., were prepared* from bovine albumin and human gamma globulin. The same series of concentrations was prepared for each of the following albumin-globulin ratios: 1/0, 8/1, 4/1, 1/1, 1/4, 1/8, 0/1. In all, 49 solutions representing seven different total protein concentrations for each of the seven albumin-globulin ratios were used to measure the comparative accuracy of the methods.

Determinations of total protein content of each solution were then made by the colorimetric method and by two turbidimetric methods (original VDRL and modified VDRL). The spectrophotometer used was a Coleman Universal, model 11, with turbidimetric readings made at 420 and colorimetric at 750 millimicrons. Since the test solutions were relatively pure protein solutions, the colorimetric method was applied to the solutions without the use of the correction factor recommended by the authors for use with spinal fluids to compensate for color produced by nonprotein material. The only modification made in the colorimetric method was in the use of bovine albumin of known protein content instead of human serum for calibration of the spectrophotometer.

The investigation was further extended to show the influence upon results of the manner of calibration of the instrument used for measuring light transmission in the modified VDRL

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method. For that purpose, three different "standard" curves were used for conversion of light transmission readings into milligrams of total protein—one based upon use of pooled human blood serum as standard protein; another, upon use of a mixture of four parts albumin with one part of gamma globulin; the third, upon use of a mixture of equal parts of albumin and gamma globulin.

RESULTS

The results obtained are shown in Table I. The colorimetric method yielded results very close to the Kjeldahl-controlled concentrations throughout the entire range of ratios and concentrations; the original VDRL method was less accurate, yielding low values when the test solutions were predominantly globulin; the modified VDRL method yielded values which ranged from low figures to high figures for each concentration in direct proportion to the magnitude of the A/G ratio. The greatest deviation of the colorimetric method from

TABLE I. TOTAL PROTEIN DETERMINATIONS BY FIVE METHODS ON KJELDAHL-CONTROLLED SOLUTIONS OF KNOWN CONCENTRATIONS AND A/G RATIOS

METHOD	A/G ALL 7 RATIOS	MG. TOTAL PROTEIN PER 100 ML. (SINGLE DETERMINATIONS)						
KJELDAHL		10	20	40	60	80	100	140
	1/0	11	22	46	62	83	105	145
	8/1	10	22	46	60	80	105	145
	4/1	9	22	45	62	83	102	140
A	1/1	10	23	43	62	80	102	145
	1/4	9	23	43	62	82	100	145
	1/8	9	21	42	62	82	100	145
	0/1	9	21	42	60	81	100	145
	1/0	9	17	34	56	76	98	132
	8/1	9	17	34	56	74	94	132
	4/1	9	16	34	56	74	94	134
B	1/1	9	16	34	56	74	94	132
	1/4	8	15	32	56	76	86	128
	1/8	7	15	32	56	70	86	122
	0/1	7	14	32	48	74	86	122
	1/0	23	30	76	130	211	240	257+
	8/1	13	28	70	100	193	211	257
	4/1	11	21	47	84	160	176	211
C	1/1	10	19	40	57	88	104	156
	1/4	8	17	32	42	70	78	118
	1/8	8	15	25	45	65	73	107
	0/1	6	11	25	36	55	67	94
	1/0	20	26	64	106	172	200	230
	8/1	13	24	60	85	120	188	230
	4/1	10	20	40	72	107	153	188
D	1/1	8	17	35	49	76	86	134
	1/4	7	15	27	37	67	74	100
	1/8	7	12	23	39	60	62	90
	0/1	5	9	23	32	36	58	79
	1/0	18	24	54	80	125	150	200
	8/1	15	22	52	68	136	170	200
	4/1	11	19	36	60	92	107	136
E	1/1	7	16	31	43	60	69	84
	1/4	6	15	25	33	60	66	75
	1/8	6	12	20	35	51	53	70
	0/1	5	9	20	28	35	46	64

Method: A = Colorimetric (Daughaday and associates).
B = Original VDRL turbidimetric.
C = Modified VDRL; calibration with protein of A/G ratio 1/1.
D = Modified VDRL; calibration with pooled human serum.
E = Modified VDRL; calibration with protein of A/G ratio 4/1.

TABLE II. ANALYSIS OF EXTENT OF DEVIATION FROM KJELDAHL VALUES SHOWN BY EACH METHOD OVER ENTIRE RANGE OF A/G RATIOS

	RESULT COMPARED WITH KJELDAHL VALUE	METHOD				
		A	B	C	D	E
No. results in range indicated	Higher by more than 15%	0	0	19	18	12
	Not more than 15% difference	49	37	11	8	9
	Lower by more than 15%	0	12	19	23	28

the Kjeldahl value did not exceed 15 per cent, and only five of the 49 determinations deviated by more than 10 per cent. Deviations for the other methods are shown in Table II.

DISCUSSION

The results obtained on protein solutions throughout the entire range of A/G ratios suggest that the colorimetric method for the determination of total protein investigated is superior to turbidimetric methods using trichloracetic acid as a protein precipitant. Since trichloracetic acid has been shown by others[2] to be a better reagent under comparable conditions than sulfosalicylic acid, it follows that the colorimetric method is superior to any recommended turbidimetric method.

The superiority of the colorimetric method is most marked when a preponderance of gamma globulin over albumin is present in the material examined. However, this superiority is evident to some extent throughout the entire range of A/G ratios, and is reflected by the fact that the colorimetric method yielded results deviating by not more than 10 per cent from values established by Kjeldahl determinations in 44 of 49 tests, while the original VDRL method showed only 24 of the 49 determinations within that range. In addition, none of the results by the colorimetric method deviated from the Kjeldahl determination by more than 15 per cent, whereas the original VDRL method deviated by 20 per cent in seven tests, 25 per cent in two tests, and 30 per cent in three tests on solutions with A/G ratios of one-fourth or less with total protein concentrations of 60 mg. per 100 ml. or less. Deviations of determinations by the modified VDRL method were even greater, were directly proportional to the magnitude of the A/G ratio, and were affected by the A/G ratio of the standard protein solution used for calibration of the spectrophotometer.

These considerations suggest that the colorimetric method investigated is entirely suitable for the determination of total protein in spinal fluid. In fact, it appears to be the method of choice. Because it can be performed with a commendable degree of accuracy on 0.2 ml. of specimen, it is ideally suited for use in a public health laboratory.

SUMMARY AND CONCLUSIONS

The colorimetric method for total protein in spinal fluid recommended by Daughaday, Lowry, Rosebrough, and Fields[3] and based upon the use of Folin-Ciocalteau reagent in the presence of copper has been shown to be relatively unaffected by the albumin-globulin ratio of the protein present in contradistinction to accepted turbidimetric methods. The method is, therefore, preferable

to turbidimetric methods; its value is enhanced by the fact that it permits a high degree of accuracy in determinations made on quantities of specimen as small as 0.2 ml. It is, however, desirable to base calibrations of instruments used upon results with a standard protein of known concentration rather than upon the use of pooled serum or plasma.

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A DIRECT METHOD FOR THE DETERMINATION OF EVANS BLUE USING ZEPHIRAN AS A SOLVENT

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INTRODUCTION

A NUMBER of analytical difficulties are attendant upon the study of plasma volume by the Evans Blue dilution method in small laboratory animals following total body x-irradiation. Only small blood samples are available, and the post-irradiation samples are frequently hemolyzed and very lipemic. The procedure described here requires only 0.020 ml. of plasma for each determination, has proved satisfactory even under extreme conditions of lipemia and hemolysis, and avoids the dye adsorption errors described by Caster, Simon, and Armstrong.[1] The method involves the direct spectrophotometric determination of Evans Blue in dyed plasma samples using an aqueous zephiran chloride solution as a solvent.

MATERIALS AND METHODS

Solutions.—Standards—A 1 per cent solution of Evans Blue in distilled water is filtered through a sintered-glass filter and stored in a glass bottle. This solution is tested for purity,[3] and a 100 γ/ml. stock solution is prepared. Injection solutions and standards for the spectrophotometric determination are prepared directly from this 100 γ/ml. stock solution. Routinely, standards of 0, 2, 4, 6, 8 and 12 γ/ml. of dye prepared in zephiran are read with each series of plasma unknowns.

Zephiran—The commercially available 12.8 per cent solution of zephiran chloride is used directly as a solvent without further dilution or purification. Manipulative difficulties due to foaming are avoided by using Krogh-Keys pipettes[4] for measuring and transferring this solution.

Procedure.—20 μl of fresh heparinized plasma is pipetted into a 3 by 10 mm. microcell for a Beckman DU spectrophotometer with a constriction pipette,* and 200 μl of zephiran solution added with a Krogh-Keys pipette.[4] These solutions are mixed thoroughly by inserting a vibrating Pyrex fiber† into the cuvette. Optical density readings are obtained at 400 mμ and 620 mμ, using distilled water as the photometric blank. Unknown dye concentrations are obtained by a comparison with the standards. Since Beer's Law was obeyed in all cases,[1] a constant is used in the computation. In this procedure there is no difference in K-value with different plasma samples. With two different spectrophotometers employing different slit widths, constants of 0.072 and 0.075 optical density/γ of dye per milliliter, were found.

Blank.—Variations in plasma blank provide a substantial source of uncertainty in plasma volume estimations by the Evans Blue dilution method. These variations occur as a result of: (a) varying degrees of hemolysis, (b) varying amounts of lipemia, and (c) variations in plasma blank which are independent of hemolysis and lipemia (Table II). Of these, hemolysis and lipemia are the most critical sources of error.

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*Linderstrom-Lang and Holter micro volumetric pipettes available at A. H. Thomas Co., Philadelphia, Pa.

†Magnetic stirrer available at Microchemical Specialties Co., Berkeley, Calif.

Hemolysis Correction.—The following formulas have been used in our work to compensate for variations in the extent of hemolysis in the different samples. An advantage over the previously used correction formula is obtained in that hemoglobin-free plasma samples are not required for comparison.[2] To avoid this restriction we have based our calculations upon the use of distilled water as an optical blank for all spectrophotometric readings.

Prior to injecting the dye, a plasma sample is obtained from each animal. A "blank" value is calculated from this sample. To do this, the sample is diluted with zephiran solution and spectrophotometric readings are made at 400 and 620 mμ against distilled water as a photometric blank. In zephiran solution, the usual 540 mμ peak of hemoglobin is absent. The 400 mμ reading is used to compute the hemoglobin correction. The blank value is computed from the following formula:

$$A_{620} - \frac{A_{400} - Z}{F_1} = B_{620} \qquad (1)$$

where:

A_{620} and A_{400} = the observed absorption readings obtained at 620 mμ and 400 mμ, respectively.

Z = the absorption at 400 mμ of a distilled water blank made up in zephiran solution when read against distilled water as a photometric blank. This factor can be eliminated if a zephiran solution is used as the photometric blank for all determinations. We initially felt that distilled water was a safer and more repeatable blank material than zephiran. The latter liquid has proved surprisingly constant in practice. In our instruments Z has the constant value of 0.034.

$F_1 = H_{400}/H_{620}$, where H_{400} and H_{620} represent the absorption readings due to hemoglobin at 400 mμ and 620 mμ, respectively.

B_{620} is the desired plasma blank value at 620 mμ. It is assumed that this preinjection B_{620} remains constant throughout the experimental period.

At set periods following the injection of the dye, plasma samples are obtained from the animal. These are diluted with zephiran as described above, and spectrophotometric readings made at 400 mμ and 620 mμ. The absorption at 620 mμ due to the dye was calculated from the formula:

$$\frac{F_1F_2}{F_1F_2-1} A_{620} - \frac{F_2}{F_1F_2-1} (A_{400}-Z) - B_{620} = D_{620} \qquad (2)$$

where:

B_{620} = corrected blank reading at 620 mμ for the preinjection blank calculated from formula (1).

$F_1 = H_{400}/H_{620}$, i.e., the ratio of the absorption of hemoglobin in zephiran at 400 mμ compared with that at 620 mμ.

$F_2 = D_{620}/D_{400}$, i.e., the optical density of the dye (Evans Blue) in zephiran at 620 mμ divided by its optical density reading at 400 mμ.

The exact values of F_1 and F_2 vary with instrument. Two different Beckman spectrophotometers employing slit widths of approximately 1.0 and 0.2 mm. at 620 mμ yielded values of 13 and 10, respectively, for F_1, and 8 and 10 for F_2. There seem to be slight changes in these values from time to time, depending upon slit width. In Table I, for example, the values of F_1 and F_2 are found to be closer to 12 and 9, respectively.

The above formulas can be derived from the following considerations. Assume that the absorbing system is composed of hemoglobin, Evans Blue, blank, and zephiran. In the preinjection sample the observed reading at 620 mμ minus the absorption due to hemoglobin gives the calculated blank, or:

$$A_{620}-H_{620}=B_{620} \qquad (3)$$

but:

$$H_{620} = \frac{H_{400}}{F_1} \simeq \frac{A_{400}-Z}{F_1} \qquad (4)$$

TABLE I. USE OF HEMOLYSIS CORRECTION FORMULA TO CORRECT FOR THE EFFECT OF VARYING AMOUNTS OF HEMOLYSIS UPON THE SPECTROPHOTOMETRIC READINGS AT 620 mμ

% HEMOLYZED PLASMA*	DYE CONC. γ/ML.	OPTICAL DENSITY† AT		CORRECTED OPTICAL DENSITY AT 620mμ CALCULATED BY	
		400 mμ	620 mμ	SUBTRACTION‡	FORMULA
0	Blank	0.180	0.017		0.006
	1.82	0.223	0.156	0.139	0.135
	3.64	0.224	0.281	0.264	0.261
	5.45	0.237	0.408	0.391	0.388
17%	Blank	0.540	0.045		(0.006)
	1.82	0.563	0.170	0.125	0.121
	3.64	0.588	0.305	0.260	0.256
	5.45	0.583	0.425	0.380	0.378
33%	Blank	0.905	0.069		(0.002)
	1.82	0.960	0.200	0.131	0.121
	3.64	0.960	0.330	0.261	0.252
	5.45	0.975	0.458	0.389	0.381
67%	Blank	1.62	0.139		(0.017)
	1.82	1.61	0.266	0.127	0.137
	3.64	1.62	0.386	0.247	0.258
	5.45	1.63	0.511	0.372	0.383
Standards (no plasma)	Blank	0.034	0.000		
	1.82	0.050	0.132	0.132	0.132
	3.64	0.064	0.260	0.260	0.261
	5.45	0.078	0.385	0.385	0.386

*The plasma samples used were mixtures of normal plasma to which a severely hemolyzed plasma was added in the proportions shown.
†Blank, samples, and standard solutions were prepared in zephiran. All readings are in relation to distilled water as the photometric blank.
‡The "subtraction" values are given only as a check on "formula" values. These "subtraction" values rely upon in vitro blanks which are not available in in vivo work.

by substituting this value of H_{620} into equation (3) we obtain equation (1) directly.

After the dye has been injected:

$$A_{620} - D_{620} - H_{620} = B_{620} \tag{5}$$

When dye is present, equation (4) takes the form:

$$H_{620} = \frac{H_{400}}{F_1} = \frac{A_{400} - \frac{D_{620}}{F_2} - B_{400} - Z}{F_1} \tag{6}$$

where B_{400} is the corrected blank reading at 400 mμ.

Substituting this value of H_{620} into equation (5) and solving for D_{620}, we find:

$$D_{620} = \frac{F_1F_2}{F_1F_2-1} A_{620} - \frac{F_2}{F_1F_2-1} (A_{400} - B_{400} - Z) - \frac{F_1F_2}{F_1F_2-1} B_{620} \tag{7}$$

or:

$$B_{620} \simeq \frac{F_1F_2}{F_1F_2-1} A_{620} - \frac{F_2}{F_1F_2-1} (A_{400} - Z) - B_{620}$$

In practice the terms $\frac{F_1F_2}{F_1F_2-1}$, $\frac{F_2}{F_1F_2-1}$, and Z are constants, and the computations thus become relatively simple.

In Table I the results obtained, when these formulas were applied to a set of experimental data, are seen. The various plasma samples were prepared by mixing two plasma samples. One was a normal-appearing plasma and the second was a grossly hemolyzed plasma (prepared by freezing a mixture of plasma and red cells) containing nearly 1 per cent hemoglobin. Since these solutions were made up in vitro it was possible to obtain true plasma

TABLE II. NORMAL VARIATION IN THE BLANK READING AT 620 Mμ

	SAMPLE NO. *	400 Mμ	620 Mμ	CALCULATED BLANK AT 620 Mμ†
Rat 1	1	0.680	0.066	0.016
	2	0.910	0.087	0.013
	3	0.374	0.017	−0.009
Rat 2	1	0.505	0.067	0.031
	2	0.340	0.039	0.016
	3	0.484	0.058	0.023
Rat 3	1	2.34	0.334	0.156
	2	2.57	0.305	0.109
	3	0.707	0.072	0.020
Rabbit	1	0.370	0.056	0.030
	2	0.580	0.067	0.025
	3	0.356	0.055	0.030

*Blood samples numbered 1, 2, and 3 were withdrawn from the animal's heart at consecutive one-hour intervals.
†Calculated with the aid of formula (1).

blank readings for each set of dyed plasmas. This fact allowed the calculation of the dye absorption at 620 mμ by two procedures. In the fifth column of this table the values shown were calculated directly by subtracting the corresponding blanks. The values in the sixth column of Table I were calculated through the use of the above formulas using the first plasma sample (0 per cent hemolyzed plasma in normal plasma) as the B_{620}. The calculations were based upon this initial plasma blank value because this corresponds more nearly to the in vivo situation in which these latter blank values cannot be obtained. The calculated blank values for each of the other plasma mixtures are given in parentheses. They were not used in these calculations but are presented to show the small size and the constancy of the B_{620} obtained in this manner. It is seen that in spite of the fact that the undyed plasma absorption readings at 620 mμ undergo a nearly tenfold change, the values in column 6 are in agreement both with the values in column 5 and with the standard values given at the end of the table.

If the optical density reading at 400 mμ is greater than 1.5 to 2.0, it is often difficult to obtain a useful correction value. A 400 mμ reading can be obtained by shifting the sensitivity of the spectrophotometer (as was done for the higher readings in Table II), or by diluting the sample with additional amounts of zephiran. As is seen from the results with rat 3 in Table II, however, substantial errors may occur when corrections become excessively large. Such extreme values were rarely encountered.

APPLICATION

Lipemia.—In Table III are shown the plasma blank and dyed plasma readings obtained on a normal sample and on a grossly lipemic sample. The saline values were calculated by direct subtraction. Under normal working conditions,

TABLE III. EFFECT OF LIPEMIA UPON THE DETERMINATION OF EVANS BLUE

SAMPLE	READINGS WITH SALINE AS DILUENT‡			READINGS WITH 10% ZEPHIRAN AS DILUENT			
	620 Mμ	BLANK	DYE	400 Mμ	620 Mμ	BLANK§	DYE§
Control plasma*	0.016	0.016		0.198	0.020	0.007	
Control plasma plus dye†	0.260		0.244	0.237	0.339		0.317
Lipemic plasma	0.063	0.063		0.314	0.020	−0.002	
Lipemic plasma plus dye	0.314		0.251	0.344	0.325		0.303

*"Control plasma" samples were taken before the administration of corn oil and "lipemic plasma" samples were taken one hour thereafter.
†Dyed plasma samples were prepared in vitro.
‡Saline blank values are shown for comparison with the blank values observed in zephiran.
§Calculated with aid of the formulas in the text.

of course, this cannot be done, and very large errors would occur. The zephiran values were calculated by the above formulas without making use of the in vitro blanks.

In spite of the fact that the zephiran calculations were restricted to values obtainable under normal working conditions while the saline figures made use of in vitro blanks, the agreement between the dye values in zephiran was of the same order as that in saline (4 per cent of the control value in zephiran as compared with 3 per cent in saline). Using the control blank as the only available blank for the saline figures, a 22 per cent error would have been obtained.

In terms of absolute error, the difference between normal and lipemic plasma blanks was five times as great when read in saline as when read in zephiran solution. The difference between the zephiran blank of normal and lipemic samples was hardly greater than would be expected from two different plasmas drawn an hour apart (see Table II).

Manipulative Procedures.—It has been shown[1] that adsorption errors are most critical in dilute solution. The 100 γ/ml. stock solution can be handled and stored with negligible changes for prolonged periods of time. Where precise standardization is required over a period of time, it is well to allow this solution to stand for a week or so prior to standardization.

TABLE IV. PLASMA VOLUME OF RABBITS BEFORE AND AFTER TOTAL BODY X-IRRADIATION

RABBIT NO.	CONTROL PERIOD (WEEKS)			PRE-X-RAY AVERAGE	POST X-IRRADIATION		SHAM IRRADIATED	DIFFERENCE*
	1ST	2ND	3RD		12 HRS.	36 HRS.		
9	3.00[1]	2.95	3.10	3.02		---		
10	3.32	3.67	3.42	3.47	3.86			+0.39
11	4.00	3.71	3.51	3.74	4.04			+0.30
12	3.90	3.87	3.72	3.83	4.27			+0.34
13	2.82	3.09	3.14	3.02		4.07		+1.05
14	3.41	3.61	3.33	3.45			3.46	+0.01
15	4.14	4.32	3.89	4.12			3.92	–0.20
16	3.97	4.02	3.58	3.86		4.92		+1.06

*All values are expressed as a per cent of body weight.

When more dilute solutions of dye must be handled, particular care must be taken to avoid errors due to adsorption on the surfaces of pipettes, syringes, and other glass containers. It has been shown[1] that nearly 40 per cent of the dye adsorption on glass surfaces occurs within the first minute or two. To eliminate the adsorption errors in pipetting and injecting dilute dye solutions, one should fill the pipette or syringe with the solution to be measured, allow it to stand for two to three minutes, eject this solution, and then refill the pipette or syringe with another aliquot of the solution to be measured.

Adsorption of dye on the wall of volumetric flasks is usually a trivial error provided the dye concentration is high. Adsorption can be eliminated, of course, by diluting directly in zephiran solution. At dye concentrations greater than about 20 γ/ml., however, these zephiran-containing dye solutions are not stable. On standing several days, the absorption reading of a 100 γ/ml. dye solution at 620 mμ decreases by many fold, and reddish crystals appear. This is appar-

ently due to a complex formation, since when these red crystals are washed with chloroform, a blue residue is found which appears to be Evans Blue.

Precision.—In our plasma volume studies, plasma Evans Blue concentrations are routinely determined in duplicate. A random sample of 100 of these pairs of Evans Blue values was picked as a basis for estimating replicability under routine conditions. The variation between duplicates was $\frac{\sigma \times 100}{\text{mean}}$ = 2.3 per cent, where the average concentration of dye in the cuvette was 5.5 γ/ml. and the average absorption reading was 0.409. The average plasma blank absorption reading was 0.012 ± 0.014 for rats and 0.014 ± 0.008 for rabbits.

A typical sample of plasma volume data for the rabbit is shown in Table IV. The consistency of the control data for these animals furnishes an estimate of the over-all accuracy of the method. By analysis of variance it was found that, during the control period, the rabbit-by-weeks variation (interaction term) corresponded to an absolute variation of $\sigma = 0.16$ per cent where the average plasma volume was 3.56 per cent of the body weight. This amounts to a relative error of 4.5 per cent. This 4.5 per cent error includes such factors as analytical errors, injection errors, samples handling errors, and the physiologic variability in plasma volume from week to week.

Following x-irradiation there were small but consistent increases in plasma volume. By the t-test for paired variates, the changes at both periods are significant ($P < .01$) even though the number of data is small. The size of these changes in absolute terms is shown in the last column of Table IV. These highly consistent, stepwise changes in the postirradiation data are of particular interest, since it is under these conditions that severe lipemia and hemolysis are frequently present and might be expected to contribute to a large random error in the determination.

SUMMARY

(1) A direct method for the determination of Evans Blue is described which (a) requires as little as 0.020 ml. of plasma, (b) automatically eliminates most errors due to lipemia, (c) does not require individual K-values for different plasma samples, and (d) avoids the dye adsorption errors described by Caster and associates.[1]

(2) A hemolysis correction formula is presented which does not depend upon comparison with a hemolysis-free plasma sample.

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